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PHARMACOLOGICAL PROPERTIES OF 3-PHENYL-5β DIETHYLAMINOETHYL-1,2,4-OXADIAZOLE

BY

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The general pharmacological properties of Oxolamine (3-phenyl-5β-diethylaminoethyl-1,2,4-oxadiazole) are described. The antitussive activity of this drug is more apparent in tests involving a diffuse stimulation of the bronchial tree than with electrical stimulation of the superior laryngeal nerve. These results suggest a predominantly peripheral mechanism of action. Oxolamine also possesses analgesic–anti-inflammatory, local anaesthetic and antispasmodic properties. The acute and chronic toxicities of Oxolamine are low, and the experimental results indicate the absence of side effects. The possibility that the antitussive activity is related to the other pharmacological properties is discussed.

Previous investigations (Silvestrini & Pozzatti, 1960) have shown that certain substituted aminoalkyl-1,2,4-oxadiazoles belonging to a homogeneous series (Palazzo, Strani, Tavella & Silvestrini, unpublished results) possess antitussive action. The most active compound among these derivatives, 3-phenyl-5β-diethylaminoethyl-1,2,4-oxadiazole (Oxolamine), was therefore submitted to a more detailed investigation.

METHODS

In the following tests mice were of the CF1 strain and weighed from 18 to 22 g; rats were of the Long-Evans strain and weighed from 150 to 200 g. Animals of both sexes were used. Unless otherwise stated, drugs were administered in aqueous solution as the hydrochloride.

Acute toxicity. The LD50 was calculated by the method of Weil (1952) on the basis of the death rate over 5-day periods following administration of the drug.

Chronic toxicity. One-month-old rats received daily 100 mg/kg Oxolamine hydrochloride injected intraperitoneally or 500 mg/kg Oxolamine citrate administered orally. The effect on the body weight was taken as the criterion of toxicity; the numbers of red and white corpuscles, haemoglobin concentration and behavioural changes were also noted. The animals were killed and various organs were taken for histological examination.

Acute behavioural effects. Mice received 25, 50, 75, 100 and 200 mg/kg Oxolamine hydrochloride by intraperitoneal injection. Effects on locomotor activity, muscle tone, righting reflex and on autonomic functions were assessed by the method of Irwin (1959).

Similar experiments were carried out on cats weighing from 2.0 to 2.5 kg.

Local irritation. Rats received a subcutaneous injection of Oxolamine (1 mg in 0.1 ml.) in the morning, afternoon and again next morning, and were sacrificed the following afternoon.

The same dose was injected into subcutaneous tissue of the external ear of rabbits which were observed for 3 days.

Antitussive activity. The acrolein inhalation test was used (Silvestrini & Maffii, 1959; Silvestrini & Pozzatti, 1960). The guinea-pigs were placed in pairs inside a transparent box, and 8 ml. of air saturated with acrolein vapour was blown into it. Each pair consisted of a control and of a treated animal. The number of coughs was counted over a 5-min period. The animals were removed and left for 3 min, after which they were observed for a further 5 min in which the number of coughs was again counted.

In a second set of experiments guinea-pigs were exposed for 5 min to a 2.8% (w/v) ammonia aerosol (Winter & Flataker, 1954). The guinea-pigs were selected according to the number of coughing fits observed during a single aerosol administration 6 hr before the test, and all animals with more than 16 fits or less than 4 were discarded. The drugs under examination were administered intraperitoneally 30 min before the tests were started. Weak coughing fits were given a value of 0.5 and strong fits of 1.

Cats were anaesthetized with pentobarbitone sodium (35 to 50 mg/kg intraperitoneally). The superior laryngeal nerve was stimulated electrically by the method of Domenjoz (1952). The drug was administered intravenously at the rate of 10 mg/kg in 2 min.

Analgesic–anti-inflammatory action. The method of Randall & Selitto (1957) was used in rats which had been starved overnight.

Analgesic activity. The hot-plate (Woolfe & Macdonald, 1944) and the phenylquinone (Siegmund, Cadmus & Lu, 1957) tests were applied to mice.

In the hot-plate test equal parts of acetone and ethylformate were brought to boiling point (55°) in a cylindrical container. This test was carried out only on animals which reacted to pain within 8 to 12 sec. All subsequent readings in the same animals were made 20, 40 and 60 min after the drugs had been administered. In the second test the compounds were injected 30 min before phenylquinone [0.25 ml. per animal of a 0.02% (w/v) phenylquinone solution in 5% (v/v) ethyl alcohol].

Local anaesthetic activity. This was tested in rabbits by finding the least concentration required to abolish the corneal reflex induced by touching the cornea (10 times consecutively) with a horse hair (Sollmann, 1918). Three drops of a buffered solution (*p*H 7) were instilled into the conjunctival sac, 3 min before the test.

Antispasmodic action in vitro. Strips of guinea-pig intestine were suspended in oxygenated Tyrode solution at 37° C. The dose of drug was found which caused a 50% inhibition of the contraction produced by acetylcholine chloride (0.02 μg/ml.), histamine hydrochloride (0.2 μg/ml.), barium chloride (50 μg/ml.) and dimethylphenylpiperazinium iodide (2 μg/ml.).

The tracheal chain preparation described by Castillo & De Beer (1947) was also employed. The contractions were induced with acetylcholine (0.5 μg/ml.).

Effect on the arterial blood pressure, respiration and on the response of the nictitating membrane. Adult cats were anaesthetized with chloralose (70 mg/kg) and pentobarbitone sodium (5 to 10 mg/kg), both given by intraperitoneal injection. The blood pressure was recorded with a mercury manometer connected to a cannula in the femoral artery. Respiration was recorded by a Marey tambour connected to the trachea. The effect of the drug was followed on the response of the blood pressure to doses of adrenaline (1 to 5 μg/kg intravenously), acetylcholine (0.5 μg/kg intravenously), dimethylphenylpiperazinium iodide (10 to 20 μg/kg intravenously) and to electrical stimulation of the distal end of the vagus cut in the neck. The response of the nictitating membrane was obtained by preganglionic stimulation of the cervical sympathetic nerve trunk (duration 4 min; frequency 10 to 30 sec; rectangular pulses of 1 msec duration).

The effect of the drug was also studied on the blood pressure of the unanaesthetized rat. A transistor microphone and air coupling were used whereby the pressure was progressively

increased until the blood pulsation at the tail ceased. The apparatus was designed and constructed in the workshop of the authors. It consists of a transistor amplifier of very low frequency response, and has filters to ensure non-sensitivity to all outside noise. A charcoal microphone was connected to the tail by means of a screw-graduated forceps.

Effect on intestinal peristalsis. The method was that of Stickney, Northup & Van Liere (1955). Mice were starved for 20 hr before the test. The drug was administered orally 30 min before the charcoal feed (5% w/v bone charcoal in 5% (w/v) aqueous solution of gum acacia). One hour later the mice were killed and the length of intestine traversed by the charcoal was measured.

Alarm reaction. Rabbits weighing from 2.3 to 2.5 kg were used. The hypothalamus was stimulated by the method of Monnier & Laue (1953), using a Grass stimulator model S4E (duration 3 sec; frequency 250/sec; pulse 1 msec).

Effect on conditioned response. The technique described by Cook & Weidley (1957) for the pole-climbing response in rats was employed. The buzzer was continued for 15 sec, or until the rat climbed the pole.

Anti-inflammatory activity. This was studied by the method of Randall, Selitto & Valdes (1957). Rats with about the same limb volume were specially selected for the test. Oedema was produced by the injection of brewer's yeast into the plantar surface of the rat's foot, and was estimated by measuring the change in volume of the limb (Buttle, D'Arcy, Howard & Kellett, 1957).

The granuloma test was also used and was carried out with cotton pellets weighing 5 mg (Meier, Schuler & Desaulles, 1950). Treatment was continued daily for one week, after which the animals were killed. Each granuloma was freed from the surrounding fatty tissue and weighed; it was weighed again after drying at 60° for 24 hr.

Antipyretic activity. The method used was that of Smith & Hamburger (1935). Sixteen hours before the test, each rat received an injection (10 ml./kg subcutaneously) of a 20% (w/v) brewer's yeast suspension. After the body temperature had remained constant for at least 1 hr, the animals were injected with the drug under test. The temperature was read every 30 min. The statistical significance of the values was calculated by the method of Wilcoxon (1949).

RESULTS

Acute toxicity

Table 1 shows the acute toxicity of Oxolamine and codeine in mice and rats.

By the intravenous route, Oxolamine and codeine were of similar toxicity; by other routes, Oxolamine was the less toxic. Oxolamine was lethal within 3 hr of administration, irrespective of the route.

TABLE 1

ACUTE TOXICITY IN MICE AND RATS

LD50 (mg/kg) with fiducial limits (*P* 0·05)

	Mouse				Rat	
Compounds	Intra-venously	Intra-peritoneally	Sub-cutaneously	Orally	Intra-peritoneally	Orally
Oxolamine hydrochloride	63 (55–72)	208 (179–241)	465 (371–583)	929 (772–1,120)	185 (138–248)	1,650 (1,370–2,000)
Oxolamine citrate	—	351 (318–387)	—	—	—	2,500
Codeine hydrochloride	67 (54–82)	125 (116–135)	286 (269–314)	365 (271–491)	—	—

Chronic toxicity

Neither Oxolamine hydrochloride (100 mg/kg) administered intraperitoneally each day for 45 days nor Oxolamine citrate (500 mg/kg orally) administered daily for 6 months affected the blood picture or body growth, or caused any pathological changes in the liver, kidney, spleen, heart or adrenals.

Local tolerance

The tests carried out on rats showed no local irritating effects. In rabbits there was a transient reddish zone at the site of injection. This local effect was less evident with Oxolamine citrate than with the hydrochloride.

Effects on behaviour

In mice, Oxolamine administered intraperitoneally in a dose of 50 mg/kg was without effect; 75 mg/kg reduced spontaneous locomotor activity and muscular strength; 100 mg/kg intensified these effects and produced ataxia; lethal doses caused convulsions.

In cats, Oxolamine, 50 mg/kg, had no apparent effect on the central nervous system or on the autonomic nervous system. The same dose of codeine provoked excitement of the sham rage type, disturbance of locomotion, mydriasis and vomiting.

The alarm reaction in the rabbit remained unaltered after 20 mg/kg Oxolamine injected intravenously. The same doses induced a transient alteration in the righting reflex. Morphine in a dose of 5 mg/kg intravenously increased the threshold stimulus.

The pole-climbing response in the rat remained unchanged after subcutaneous injection of 100 mg/kg of Oxolamine. A slightly delayed climbing time was observed, which suggests impaired muscular activity.

Antitussive action

The results are shown in Table 2.

An antitussive action was obtained with intraperitoneal injections of 1 mg/kg Oxolamine or with 2 mg/kg codeine. Oxolamine citrate produced the same inhibition of coughing as did the hydrochloride.

Oxolamine was twice as active as codeine in the test with ammonia aerosol. In this test the effective dose of both drugs was lower than that obtained with the same drugs in the acrolein test, possibly because coughing induced with ammonia aerosol was less intense. The results obtained in this series of experiments are shown in Table 3.

The action of Oxolamine on the cough reflex of the cat was as follows. After intravenous administration of 2 mg/kg a 50% inhibition was observed for 20 min in 1 of 4 animals; with 5 mg/kg the inhibition was 70 to 80% in 2 of 4 animals for 35 and 40 min respectively; and with 10 mg/kg the coughing response disappeared in 7 of 8 animals for more than 60 min.

TABLE 2

EFFECTS OF OXOLAMINE AND CODEINE ON ACROLEIN-INDUCED COUGHING IN GUINEA-PIGS

Control animals				Treated animals				
No. animals	No. coughs mean±s.e.	Compounds	mg/kg intraperitoneally	No. animals	No. coughs mean±s.e.	Inhibition %	t	p
5	15·4±2·6	Oxolamine	0·5	5	12·9±1·31	16	1·2	n.s.
15	13·0±1·27		1	15	9·26±0·88	29	3·43	0·01–0·001
16	13·93±1·52		2	16	8·53±0·72	39	3·1	0·01–0·001
10	13·8±1·29	Codeine	1	10	11·75±1·3	15	1·59	n.s.
10	14·4±1·56	hydrochloride	2	10	9·6±1·37	33	3·2	0·01–0·001

TABLE 3

EFFECTS OF OXOLAMINE AND CODEINE ON AMMONIA-INDUCED COUGHING IN GUINEA-PIGS

Number of coughs mean±s.e.

Compounds mg/kg intraperitoneally	No. animals	Before drug	After drug	Inhibition %	t	p
Saline	5	9·7±1·2	7·8±0·8	19·6	1·77	n.s.
Oxolamine hydrochloride 0·5	5	9·9±1·3	5·1±0·6	49·5	4·4	0·01–0·001
Codeine hydrochloride 1	7	8·86±2·2	4·92±0·8	44·5	3·0	0·02–0·01

TABLE 4

ANALGESIC-ANTI-INFLAMMATORY ACTIVITY IN RATS

(Randall and Selitto (1957) test)

Compounds	Doses mg/kg	No. animals	+30′ thresh. increase	t p	+90′ thresh. increase	t p	+150′ thresh. increase	t p
Oxolamine	10 subcutaneously	10	0	—	0	—	0	—
Oxolamine	20 subcutaneously	15	66	5·4 <0·001	30·7	3·37 0·01–0·001	20·7	3·58 0·01–0·001
Oxolamine	40 subcutaneously	15	58·7	2·89 0·01–0·001	53·7	3·42 0·01–0·001	29·4	1·59 n.s.
Oxolamine	40 orally	10	47	3·43 0·01–0·001	37	4·7 <0·001	28·5	4·2 <0·001
Aspirin	10 subcutaneously	5	0	n.s.	0	n.s.	0	n.s.
Aspirin	20 subcutaneously	20	42·3	4·75 <0·001	32	3·35 0·01–0·001	15·5	n.s.

Analgesic action

Results of the analgesic–anti-inflammatory test are shown in Table 4.

Oxolamine, both by subcutaneous injection and oral administration, displayed analgesic activity similar to that obtained with aspirin. However, Oxolamine in

doses up to 50 mg/kg subcutaneously was not analgesic in the hot-plate and phenylquinone tests, where morphine had been shown to be active in a dose of 2 mg/kg subcutaneously. Aspirin up to 80 mg/kg subcutaneously was without analgesic effects in the hot-plate test.

Local anaesthetic action

Oxolamine (1.0 to 1.5% w/v) produced anaesthesia of the rabbit cornea. Oxolamine citrate was as active in similar concentration.

Antispasmodic action

Oxolamine as the hydrochloride and citrate (5 μg/ml.) antagonized the effect of acetylcholine, histamine, dimethylphenylpiperazinium iodide, and barium chloride on the isolated intestine, and was about as active as papaverine in this test. In the tracheal chain preparation equiactive inhibitory doses of Oxolamine and papaverine were 2 μg/ml. and 1.2 μg/ml. respectively.

Arterial pressure and respiration

In the cat Oxolamine given intravenously in doses of 5 mg/kg produced a transient fall of the arterial pressure ranging from 5 to 10 mm Hg; 10 mg/kg caused the pressure to fall by 10 to 30 mm Hg and remain low for 3 to 8 min. There was a simultaneous increase in both the depth and frequency of respiration. Oxolamine up to 10 mg/kg did not modify the effects on the blood pressure of adrenaline, acetylcholine, dimethylphenylpiperazinium iodide, and of electrical stimulation of the vagus nerve, nor did it inhibit the contractions of the nictitating membrane.

In the conscious rat, Oxolamine given intraperitoneally up to 20 mg/kg did not modify the arterial pressure.

Action on intestinal motility

The results are given in Table 5.

Oxolamine had no effect in doses up to 50 mg/kg orally. Atropine (5 mg/kg) and codeine (25 mg/kg) delayed the progression of the charcoal meal.

TABLE 5
EFFECT ON PROGRESSION OF CHARCOAL MEAL IN MICE

Compounds mg/kg orally	No. animals	A Intestine length (cm) mean±s.e.	B Progression of charcoal (cm) mean±s.e.	Percentage of B/A	Comparison with controls t	Comparison with controls p	% Inhibition
Saline	20	28·6±0·6	18·8±1·27	65·7	—	—	—
Oxolamine 50	15	29·7±0·94	21·1±1·1	71	—	n.s.	0
Codeine 25	15	26·9±2	13±1·4	48·3	3·06	0·01–0·001	30·9
Codeine 50	5	28±1·56	11·9±2·6	42	2·45	0·05–0·02	37·3
Atropine 2·5	5	29·8±1·33	16·9±2·8	56·7	0·6	0·5–0·6	20·1
Atropine 5	5	27·1±1·3	6·4±0·8	23·6	4·7	<0·001	66

TABLE 6
ANTI-INFLAMMATORY ACTIVITY IN RATS

Compounds	Doses mg/kg subcutaneously	No. animals	Reduction of limb volume in ml. 2 hr	t p	4 hr	t p	6 hr	t p	24 hr	t p
Oxolamine	20	20	0·117	1·8 n.s.	0·127	1·75 n.s.	0·154	1·9 n.s.	0·049	n.s.
Oxolamine	40	20	0·157	2·12 <0·05	0·130	1·9 n.s.	0·110	n.s.	0·110	n.s.
Oxolamine	80	5	0·302	6·14 <0·001	0·198	7·2 <0·001	0·134	2·9 <0·002	0·080	n.s.
Phenyl-butazone	10	10	0·085	n.s.	0·098	n.s.	0·083	n.s.	0·035	n.s.
Phenyl-butazone	20	10	0·137	5·3 <0·001	0·132	2·49 <0·05	0·152	3·2 <0·01	0·167	n.s.
Phenyl-butazone	40	10	0·236	6·8 <0·001	0·274	3·8 <0·01	0·148	4·13 <0·01	0	—
Aspirin	60	20	0·030	n.s.	0	—	0	—	0	—
Aspirin	120	29	0·084	n.s.	0·178	2·28 0·05–0·02	1·188	3·6 0·01–0·001	0·067	n.s.

Anti-inflammatory and antipyretic activity

The results are shown in Table 6. The anti-inflammatory activity of Oxolamine was one-half to one-third of that possessed by phenylbutazone, but more active than that of aspirin, which, according to our experiments, required doses above 100 mg/kg subcutaneously.

Oxolamine in doses of 40 mg/kg as daily subcutaneous injections was inactive in the granuloma test in normal rats.

Oxolamine had an antipyretic action in oral doses of 200 mg/kg in rats; phenylbutazone was active at the same dose and aspirin showed an activity with 100 mg/kg orally.

DISCUSSION

The antitussive action of Oxolamine was studied in tests which differed in the type of stimulus applied to produce cough. In tests on guinea-pigs, cough was obtained by diffuse stimulation of the respiratory tract with irritant aerosols: Oxolamine was effective in small doses. In tests on anaesthetized cats, cough was obtained by electrical stimulation of the superior laryngeal nerve: Oxolamine was not effective except in large doses. The difference in sensitivity of the cough reflex to Oxolamine in the two tests suggests that the drug acts mainly on receptors in the lung and, to a lesser degree, on the cough centre. Oxolamine did not depress respiration, and in the tests on guinea-pigs it was more active than codeine.

The analgesic–anti-inflammatory action of Oxolamine, noted in the Randall & Selitto (1957) test, may be compared with that of aspirin. For example, Oxolamine showed undoubted anti-inflammatory and antipyretic activity. Oxolamine was inactive in the hot-plate test, which, according to Winder (1959), is more likely to demonstrate the analgesic activity of centrally acting drugs. Furthermore, the absence of excitation in the cat typical of morphine and codeine; the absence of

effects on the alarm reaction in the rabbit caused by central depressants and tranquillizers (Napolitano & Longo, 1957; Silvestrini, 1958; Silvestrini & Kohn, 1958), and the absence of effects on the conditioned response of the rat, such as may be seen with central analgesics (Blumberg & Dayton, 1959), strongly suggest that the analgesic action of Oxolamine is not central.

The antispasmodic action as studied on the isolated intestine of guinea-pig was unspecific, since Oxolamine inhibited contractions induced by acetylcholine, histamine, barium chloride, and dimethylphenylpiperazinium iodide. In cats, Oxolamine appeared to be devoid of antihistamine, anticholinergic, antiadrenergic, and ganglionic-blocking activity.

Oxolamine is a drug of low toxicity and would be expected to have few side-effects. Rats withstood the daily administration of a dose equivalent to one-half LD50 with no evident effect. In acute experiments, large doses of Oxolamine appeared to be without effect on behaviour, blood pressure, respiration, and intestinal motility.

The results of the present tests suggest that Oxolamine might be more effective in suppressing cough associated with inflammatory changes in the bronchial tree than cough arising exclusively from stimulation of sensory nerves in the larynx. On this view, the antitussive action would depend mainly on the anti-inflammatory and spasmolytic effect of the drug on structures in the lung, and to a lesser extent on depression of the cough centre. Further evidence, both clinical and pharmacological, is being sought to substantiate this hypothesis.

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LOCALIZATION OF CHOLINESTERASES IN THE CHICKEN NERVOUS SYSTEM AND THE PROBLEM OF THE SELECTIVE NEUROTOXICITY OF ORGANOPHOSPHORUS COMPOUNDS

BY

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Using the thiocholine method, a restricted survey has been made of cholinesterases in the spinal cord and brain stem of the chicken. No simple relation between sites of selective damage in organophosphorus neurotoxicity and centres of cholinesterase activity could be adduced. Moreover, no significant differences between species susceptible and insusceptible to poisoning by these compounds were found by this method. It is concluded that, while cholinesterase may well play an intermediary role in the intoxication, other factors determine the selective damage to certain neurones and their processes.

It is difficult at the present time to dissociate the anticholinesterase activity of certain organophosphorus compounds from being causally related to their peculiarly selective toxicity towards the nervous system of the chicken. It is, however, no longer possible to maintain the earlier view that the neuropathy and spinal tract degeneration are a direct consequence of selective pseudocholinesterase inactivation (Earl & Thompson, 1952a & b), the most important reason being the finding by Davison (1953a) that not all of those compounds which preferentially inhibit pseudocholinesterases produce delayed paralysis in chickens. On the other hand, it has been recently shown by Davies, Holland & Rumens (1960) that, so far as the di-alkyl substituted organophosphorus compounds are concerned, a relation exists between the possession by such a substance of a fluoride radicle and its ability to cause delayed paralysis. They postulated that cholinesterase might be playing an intermediary rôle in the process, combining with the inhibitor and thus releasing potentially toxic fluoride ions at critical sites.

Since it has been demonstrated that both in tri-*o*-cresyl phosphate (Cavanagh, 1954) and in diisopropyl phosphorofluoridate (dyflos) poisoning (Fenton, 1955) it is the nerve fibre which is primarily damaged and not as hitherto believed the myelin sheath, it would seem essential, if the hypothesis of Davies *et al.* (1960) is to carry any weight, to determine the topographical distribution of cholinesterases within the nervous system, paying particular regard to those centres which are damaged by the neurotoxic process. The necessity for this is particularly important

now that it has been demonstrated histochemically in the central nervous system of the rat (Koelle, 1954) that cholinesterase activity varies markedly from one centre to another. An essential prerequisite for the hypothesis mentioned above must be that an adequate quantity of cholinesterase of one type or another should be present in the areas known to be damaged. To fill this gap the present histochemical work was carried out. A brief survey of the distribution of cholinesterases in comparable areas in four laboratory mammals was also made to gain some idea as to whether the differing species sensitivity to organophosphorus compounds could have any basis in cholinesterase distribution.

MATERIALS AND METHODS

The histochemical method

Gömöri's (1952) variant of Koelle's (1950) technique with certain minor modifications was used.

Substrates. Acetylthiocholine iodide and butyrylthiocholine iodide (Light & Co.).

Selective enzyme inhibitors. Mipafox (N.N′-diisopropyl-diamidic-phosphorofluoridate); dyflos (diisopropyl-phosphorofluoridate); "62.C.47" (1:5-bis-(4 trimethyl (ammonium-phenyl) pentane-3-one diiodide); ethopropazine hydrochloride; Eserine (physostigmine). The concentrations at which these inhibitors were employed are detailed in the text and in Table 1.

Source and treatment of tissues. White Leghorn chickens 1 to 2 years old were killed by an intracardiac injection of pentobarbitone (Nembutal, Abbott). Brain stem and pieces of spinal cord with adherent dorsal root ganglia were placed in a corked pot and frozen rapidly to −70° C in an acetone/carbon-dioxide-snow mixture. The sections were cut at 10 μ in a cryostat and flattened on to albuminized slides. They were dried for 1 hr at room temperature before incubation in the reaction fluids.

Final incubation solutions

The concentrations of copper sulphate, magnesium chloride, glycine and maleate buffer in the final reaction mixture were as in Gömöri's method. Satisfactory deposits were obtained when the sodium sulphate concentration was 30% (w/v) in the acetylthiocholine mixture and 25% (w/v) in the butyrylthiocholine mixture. 25 ml. of solution was used, and the *p*H was adjusted to 6.5 before the substrate, dissolved in 1 ml. of distilled water, was mixed in. The final substrate concentration was 2 mg/ml.

In the inhibitor studies the sections were incubated for 0.5 hr at 37° C in the buffered mixture without substrate but containing inhibitor. At the end of this period the substrate in 1 ml. of distilled water was mixed in.

The sections were incubated for 3 hr at 37° C, then rinsed briefly in saturated sodium sulphate before being immersed in dilute ammonium sulphide. Sections were carried through in pairs, one of which was counterstained with alum carmine, before they were dehydrated and mounted in Canada balsam.

RESULTS

Topographical distribution of cholinesterases in the chicken nervous system

Since our interest was primarily centred upon the problem of organophosphorus neurotoxicity, only those regions which contained susceptible nerve cells or their fibres were examined. These included all levels of the spinal cord, dorsal root ganglia and spinal roots, and brain-stem and cerebellum. The last were cut together in semi-serial section at 0.5 mm intervals up to the level of the optic lobes. The diencephalon, forebrain and peripheral nerves beyond the spinal roots were not examined in this study.

The following description is based upon incubation for three hours in acetylthiocholine or butyrylthiocholine. Such anatomical terminology as is used is taken from Papez (1929).

Acetylthiocholine as substrate

Spinal cord. Enzyme activity was localized in two chief sites:

(1) All nerve cells throughout the spinal cord without exception, whether in the grey columns (Fig. 1) or lying ectopically in the white matter, contained enzyme both diffusely in their cytoplasm and in small granular masses along their surfaces.

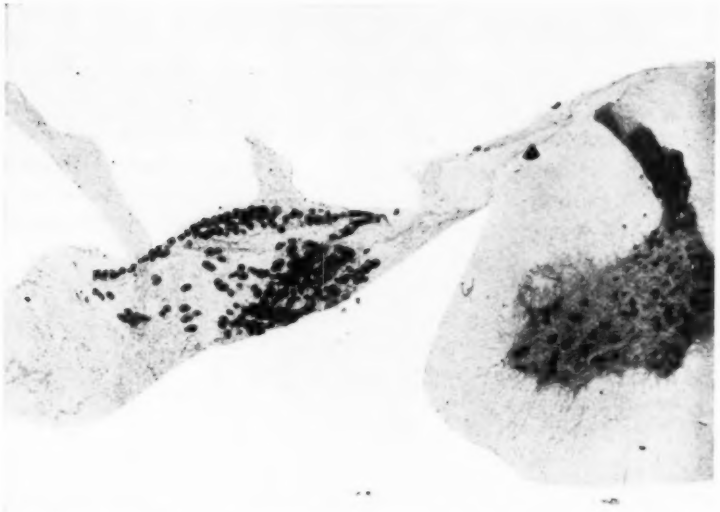

Fig. 1. Dorsal root ganglion and cervical spinal cord of chicken. Acetylthiocholine, ×21.

All cells appeared to contain an equal amount of deposit, although larger cells showed up more strikingly than smaller ones. The dorso-medial group (Clark's column) which gives rise to the spinocerebellar tracts was no different in its staining from other cell groups.

(2) The dorsal horns (Fig. 1) showed a diffuse localization of activity which was maximal in slender bands of fibres running into their medial aspect from the dorsal roots. This diffuse activity extended dorso-laterally into Lissauer's tracts and throughout the neuropil between the nerve cells. Such sites were again consistent at all levels in the spinal cord. No recognizable nerve cells were found in the dorsal caps of these horns, but small sparse cells in the intermediate zones stained exactly as nerve cells elsewhere. The fibres from the anterior grey columns extending into the white matter for short distances also stained, but no other fibre areas stained, with the exception of the fine fibres in immediate relation to ectopic subpial neurones.

Apart from these two major sites, the only other structures that stained were the walls of capillaries, but never vessels of larger calibre. No glial or ependymal cell was ever clearly stained in chicken tissues.

The nerve cells of the *dorsal root ganglia* all stained heavily (Fig. 1) and uniformly. Usually slight staining of their axons was visible.

Brain stem. Again staining occurred in two chief sites:

(1) All nerve cells stained without exception and with only minor variations in intensity (Fig. 2).

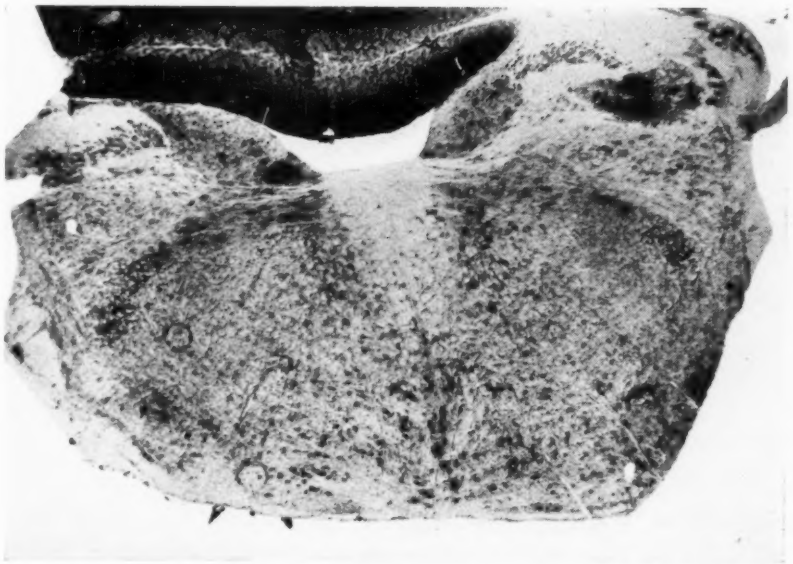

Fig. 2. Brain stem of chicken at level of lamellar nucleus. Acetylthiocholine, ×14.

(2) Well-demarcated fibre zones were stained in the following regions: (*a*) The expanded headward extension of the dorsal spinal horns in the lower medulla, and the descending root of the trigeminal nucleus; (*b*) in the vestibular and acoustic nuclear regions both medially and laterally (Fig. 2); (*c*) a small unnamed zone in the ventral pons.

Except for some irregularly crossing fibres in the pontine reticular formation, all major pathways were unstained. A conspicuously unstained fibre region was the lamellar nucleus, in the centre of which was a band of stained nerve cells. This nucleus in silver impregnated preparations has a very dense felt-work of large fibres with large synaptic terminals terminating in the band of nerve cells. Its outflow, the dorsal cochlear commissure, was also unstained (Fig. 2).

Capillaries were conspicuously stained in all regions.

Cerebellum. The molecular layer throughout stained intensely and uniformly without any discernible structural pattern. Purkinje cells showed moderate activity (Fig. 6), while the cytoplasm of the cells of the granular layer also stained moderately intensely. There was no staining of white matter.

Butyrylthiocholine as substrate

A diffuse, unlocalized, faint staining occurred in most grey and white regions of *spinal cord* and *brain stem.* Sharp localization was found in the following areas.

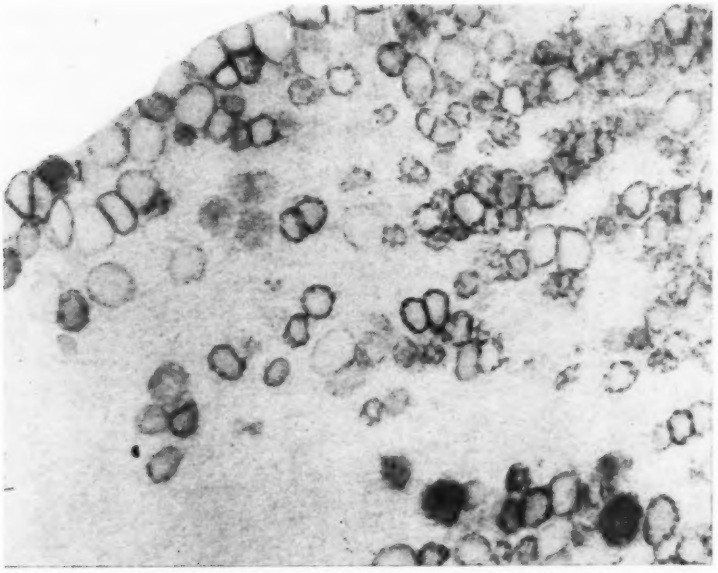

Fig. 3. Dorsal root ganglion of chicken. Butyrylthiocholine, ×82.

(*a*) Capsule cells of *dorsal root ganglia* (Fig. 3); slight staining also occurred in occasional nerve cells.

(*b*) *Choroid plexus* of fourth ventricle.

(*c*) Fibres lying in the zone between molecular and granular layers of the *cerebellum*, separating unstained Purkinje cells and sometimes running straight up into the molecular zone. These latter deposits from their position might either be part of the basket cell system around the Purkinje cell bodies, or more probably glial cells (astrocytes) which lie in this zone.

(*d*) Faint staining in the same regions that were stained when acetylthiocholine was used as substrate, that is, in fibre areas listed above and in all nerve cells. Prolonging incubation up to 6 hr caused moderate intensification of these last sites, but showed up no further structures. Evidence from the use of inhibitors and from poisoning birds with dyflos and tri-*o*-cresyl phosphate suggested that this staining was probably due to non-specific hydrolysis of butyrylthiocholine by true cholinesterase.

No staining occurred in any blood vessel, except capillaries, which, however, stained much less intensely than with acetylthiocholine and in this respect were comparable with nerve cells and fibre areas noted above. Glial cell bodies did not stain with either substrate, although it is possible that the diffuse staining seen with butyrylcholine may have been due to these cells.

Enzyme characterization by selective inhibitors

Incubation in acetylthiocholine alone provides the picture of total cholinesterase distribution because of the lack of specificity of this substrate (Holmstedt, 1957b). Furthermore, the action of eserine at 10^{-5} M concentration in abolishing all staining

with this substrate confirms that cholinesterases were responsible. Incubation in butyrylthiocholine alone, because of the specificity of this substrate towards pseudocholinesterase, should demonstrate the distribution of the latter enzyme, although since the specificity of butyrylcholine in the hen is less than it is for other species (Earl & Thompson, 1952a) doubts should be raised about this conclusion.

Prior incubation with dyflos at molar concentrations of 10^{-6}, 10^{-8} and 10^{-10} and using acetylthiocholine as substrate showed that staining in nerve cells and fibre areas was not significantly reduced at 10^{-8} M concentration, but it was abolished at 10^{-6} M concentration (Table 1). Moreover, using butyrylthiocholine all the diffuse

TABLE 1

EFFECT OF INHIBITORS ON HISTOCHEMICAL CHOLINESTERASE ACTIVITY

– No staining. + Staining significantly less than controls. ++ Staining identical with controls

		Acetylthiocholine		Butyrylthiocholine	
		Spinal cord and medulla			
Inhibitor	Molar concentration	Nerve cells	Fibre areas	Capsule cells of spinal ganglia	Cells of cerebellar cortex
Dyflos	1×10^{-6}	–	–	–	–
	1×10^{-8}	++	++	–	–
	1×10^{-10}	++	++	+	+
Mipafox	5×10^{-4}	–	–		
	5×10^{-5}	–	–		
	1×10^{-6}	+	+		
	5×10^{-7}	++	++		
	5×10^{-8}	++	++		
	1×10^{-8}	++	++		
"62.C.47"	1×10^{-3}	–	–		
	1×10^{-4}	+	+		
	2×10^{-5}	++	++		
	1×10^{-5}	++	++		
Ethopropazine hydrochloride	3×10^{-4}	+	+	–	–
	3×10^{-5}	++	++	–	–
	3×10^{-6}	++	++	++	++

staining noted with this substrate as well as the localized staining of capsule cells in spinal ganglia and the cells in the cerebellar cortex was lost at 10^{-8} M concentration of the inhibitor. Since it has been shown by Myers (1953) that pseudocholinesterase of birds is inhibited by dyflos at 10^{-8} M concentration, it is concluded that the staining from butyrylthiocholine was probably due to pseudocholinesterase activity. Conversely, most of the staining of nerve cells and fibre areas with acetylthiocholine was probably due to true cholinesterase activity, since staining was present until 10^{-6} M concentration of dyflos was used.

The action of varying concentrations of mipafox in the presence of acetylthiocholine (Table 1) confirmed that most of the staining with this substrate was probably due to true cholinesterase. Thus, no serious diminution of staining occurred at 5×10^{-7} M concentration, five times the concentration that Davison (1953b) found to block pseudocholinesterase activity in chicken brain. Staining was significantly reduced, however, at 10^{-6} M concentration and abolished at 5×10^{-6} M concentration.

The results with the reversible inhibitors " 62.C.47 " and ethopropazine, although somewhat less satisfactory, tended to support these conclusions. The latter substance in the presence of butyrylthiocholine completely abolished staining at 3×10^{-5} M concentration, but had little visible action at this concentration when acetylthiocholine was used. Bayliss & Todrick (1956) found that ethopropazine gave 99% inhibition of rat brain pseudocholinesterase at this concentration and none of the true cholinesterase, so that there may be little species difference in enzyme sensitivity to this inhibitor. The same, however, could not be said for the sensitivity to " 62.C.47." With acetylthiocholine as substrate we found that this compound only partially suppressed staining at 10^{-4} M concentration and completely suppressed it at 10^{-3} M concentration. By contrast, Bayliss & Todrick (1956) found with rat brain that true cholinesterase was inhibited 94% and 100% by 5×10^{-6} M and 2×10^{-5} M concentrations respectively. Species variation in the sensitivity of true cholinesterases to this group of substances seems to be appreciable, and it is noteworthy that Ferrari (1957) found bird cholinesterases much less sensitive than mammalian enzymes.

Results of poisoning birds with dyflos and tri-o-cresylphosphate

Two series of birds were poisoned with dyflos (0.8 mg/kg intramuscularly) and with tri-*o*-cresylphosphate (0.75 ml./kg orally). The birds treated with dyflos were protected from acute poisoning with oxime and atropine in the manner used by Davies *et al.* (1960). The effect on the histochemically demonstrable cholinesterase activity is shown in Table 2.

TABLE 2

RESPONSE TO POISONING WITH NEUROTOXIC ORGANOPHOSPHORUS AGENTS

− Negative. + Staining less than controls. ++ Staining identical to controls

		Acetylthiocholine		Butyrylthiocholine	
		Spinal cord and medulla		Capsule cells of spinal ganglia	Cells of cerebellar cortex
		Nerve cells	Fibre areas		
Dyflos 0·8 mg/kg i.m.	24 hr	++	++	−	+
	7 days	++	++	−	+
	12 days (paralysed)	++	++	++	++
Tri-*o*-cresyl phosphate 0·75 ml./kg orally	24 hr	++	++	−	−
	7 days	++	++	−	+
	12 days (paralysed)	++	++	−	+
	16 days (paralysed)	++	++	++	++

With acetylthiocholine as substrate no diminution of activity in any area at any time after poisoning was found. With butyrylthiocholine all evidence of activity disappeared from the capsule cells of the dorsal root ganglia, to return at the 12th day after dyflos and the 16th day after tri-*o*-cresylphosphate poisoning. The general diffuse staining also disappeared and returned in parallel with this localized activity. Paralysis was already present in the birds at these times. The response of the cerebellar staining with butyrylthiocholine was anomalous. One day and 7 days after dyflos there appeared to be a moderate reduction in intensity of staining,

while after tri-*o*-cresylphosphate there was no staining at 1 day and moderate staining from the 7th day onwards. Furthermore, the slight staining of nerve cells and fibre areas with butyrylthiocholine as substrate was not inhibited.

Comparison with various mammalian nervous systems

In view of the virtual restriction of the chronic neurotoxic effects of organophosphorus compounds to the chicken, cat and man, it was of interest to see whether the cholinesterase distribution was different in the chicken and cat from that of insusceptible species. The description by Koelle (1954) of the cholinesterase distribution in the rat nervous system suggested that this might be so. To this end the cervical enlargement of the spinal cord with attached dorsal root ganglia and the medulla were examined from the rat, the guinea-pig, the rabbit and the cat. The tissues were prepared exactly as were those of the chicken. The following differences were encountered in acetylthiocholine-incubated tissues, and these have been briefly referred to elsewhere by Cavanagh & Holland (1961).

Fig. 4. Guinea-pig cervical spinal cord. Acetylthiocholine, ×52.

Spinal cord. Whereas in the chicken all nerve cell bodies without exception showed cholinesterase activity, this was not true for the mammals. Enzyme activity was shown to be concentrated *around* the neurones of the ventrolateral region of the grey columns, and only slight activity was recognizable in the neuronal cytoplasm (Fig. 4). The neurones in the dorsal and medial regions of the grey column contained no cholinesterase activity or had none in their immediate vicinity. By contrast

there was always a heavy concentration of activity in the synaptic regions of the posterior horns in both the mammals and the chicken (Fig. 4). Nerve fibres emerging from the ventrolateral cell group usually had conspicuous cholinesterase activity, and especially in the rat, rabbit and guinea-pig the fibres of the ventral roots were also stained. Dorsal root fibres were never stained, although nerve cells in the ganglia showed variable cholinesterase activity. In the rat a narrow band of fibres near the midline of the posterior columns appeared to contain cholinesterase.

Brain stem. Again, in contrast to the findings in the chicken, not all the mammalian nerve cells contained cholinesterase either in their cytoplasm or in their immediate vicinity. As Koelle (1954) found in the rat, many centres are either completely devoid of or show relatively little activity. On the other hand, certain regions, considered to be synaptic areas, that were well stained in the chicken, also showed heavy staining in the mammals (Fig. 5).

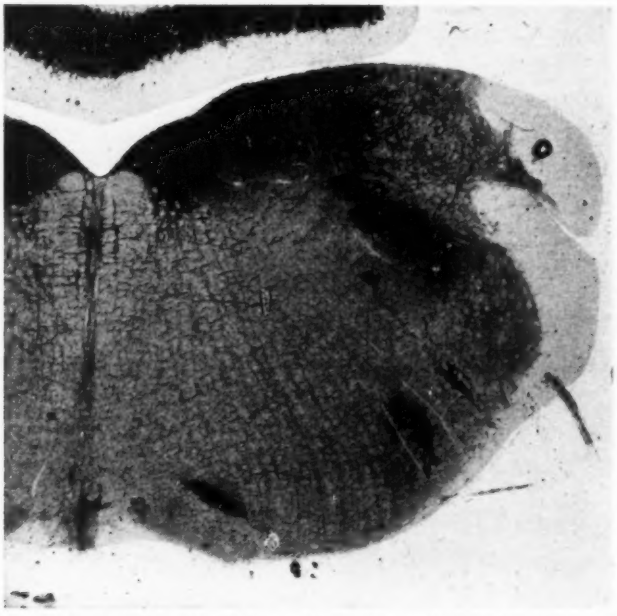

Fig. 5. Brain stem and cerebellum of rabbit. 2 hr in acetylthiocholine. Deposits in synaptic zones of vestibular areas and around cells of cranial nerve nuclei. ×12.5.

Cerebellum. The most striking differences were met with in the cerebellar cortex. The molecular (synaptic) layer was strongly positive in chicken and guinea-pig, but negative in rat, rabbit and cat. The Purkinje cells were positive in the chicken (Fig. 6) but negative in the other species. The granule cells, which stain poorly and somewhat diffusely in the chicken, were densely stained in rabbit (Fig. 6) and cat, less densely in guinea-pig and rat.

It is clear from sampling these two regions that differences of the greatest magnitude appear to exist in the cholinesterase distribution between chicken and mammals. On the other hand, certain basic similarities occur, particularly in regions of

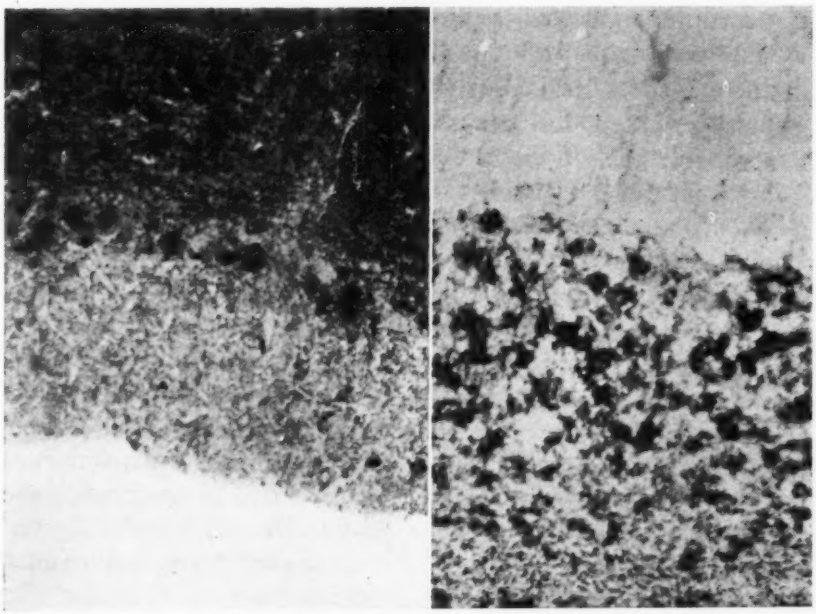

Fig. 6. Cerebellar cortex. Chicken left; rabbit right. Molecular layer at top; granular layer middle; white matter at bottom. Acetylthiocholine, ×130.

importance to the probable mechanism of organophosphorus neurotoxicity, such as the posterior grey horns of the spinal cord, which render such differences of less importance to this problem. This is also underlined by the findings in the cat, another species susceptible to organophosphorus intoxication, which does not appear greatly to differ from other mammals in its cholinesterase distribution.

DISCUSSION

The present experiments were done in order to determine whether or not a simple relation existed between distribution of cholinesterases, either true or pseudo, and the sites of damage produced by single doses of certain organophosphorus compounds. That such a relation does not exist cannot necessarily be inferred from the data presented here, although it might be fairly stated that the likelihood of its existence is not strengthened thereby. The histochemical method both for the detection of enzyme activity and for enzyme identification is fraught with uncertainties. The use of selective inhibitors, as Holmstedt (1957a) has pointed out, under conditions quite different from those used for studying enzyme characteristics by manometric methods, diminishes their value as a means of separating and identifying enzymes unless these are checked by parallel *in vitro* studies. None the less, so far as true cholinesterase is concerned, it would appear from the data given above that this enzyme is probably recognizable in all nerve cell bodies of chicken spinal cord, brain stem and cerebellum in a uniform manner, and the great variations in activity from one nuclear group to another met with by Koelle (1954) in the rat do not appear to exist in the chicken. This finding itself is of some interest to the problem of organophosphorus neurotoxicity, for should

true cholinesterase be implicated, as it may well be with phosphorofluoridates, all neurones would thus be equally at risk to their neurotoxic effects.

So far as pseudocholinesterase activity is concerned, the results obtained here were disappointing; except for localization in the capsule cells of spinal ganglia and in cells, probably glial, in the cerebellar cortex, no discrete activity was found. A similar lack of localization was found by Pepler & Pearse (1957) in the rat other than in certain hypothalamic neurones. This may in part be due to the levels of pseudocholinesterase activity, which are certainly much lower than those of true cholinesterase in the chicken nervous system (Earl & Thompson, 1952b).

The hypothesis put forward by Davies *et al.* (1960) to explain the role of cholinesterase in the neurotoxicity implies that the enzyme with which the organophosphorus compound combines should be present in the region where the subsequent biochemical lesion is produced by the liberated "toxophore" group, that is, fluoride with the phosphorofluoridates and cresolic derivatives with the aryl phosphates, for it is unlikely that such highly reactive substances would diffuse far before producing their effects. It is immaterial for this hypothesis whether true or pseudocholinesterase acts in this role, but it is particularly important to know whether the selectively damaged neurones show any difference in their cholinesterase content from those that appear to be immune.

While the question as to whether pseudocholinesterase is also present or not in neurones and other centres liable to damage is unimportant where the phosphorofluoridates are concerned, it is of some concern when the aryl phosphates are considered, for Earl & Thompson (1952a & b) have been unable to demonstrate depression of true cholinesterase activity in the chicken either *in vitro* or *in vivo* with tri-*o*-cresyl phosphate. Whether this is an absolute preference for pseudocholinesterase or whether, as with the phosphorofluoridates, the preference is relative is an important point worth re-examination. The finding of Carpenter, Jenden, Shulman & Turman (1959) that in rabbits chronically intoxicated with aryl phosphates there is a steady decline of total blood cholinesterase would suggest that the preference may be a relative one, and factors such as dose, route of administration and absorption must be taken into account. Until this point is determined it is not easy to put the aryl phosphates into line with the phosphorofluoridates.

Whether any pseudocholinesterase coexists with true cholinesterase in nerve cells or synaptic centres in the central nervous system of the chicken is difficult to determine histochemically. Holmstedt (1957b) has shown that such coexistence occurs in certain autonomic neurones of the cat, and Pepler & Pearse (1957) have demonstrated histochemically that certain neurones of the hypothalamus of the rat contain pseudocholinesterase. Where pseudocholinesterase, as demonstrated by substrate specificity and selective inhibitor concentrations, exists alone and at relatively high levels of activity such as in the capsule cells of the spinal ganglia, there is little difficulty in identifying it. Where the levels of activity are relatively low the insensitivity of the method limits its usefulness, and this probably accounts for the failure to reveal clearly pseudocholinesterase in those regions such as white matter where it is known to exist. Yet the method may not wholly be at fault, and factors such as distribution of the enzyme within the cell, species variation in

activity towards the substrate and the adequacy of the incubation medium must be taken into account. So far as our findings with each substrate and with inhibitors go, however, no evidence could be found that any of the staining in nerve cell bodies or in the synaptic areas around nerve cells or in posterior horns of spinal cord was the consequence of pseudocholinesterase activity. This does not, of course, completely exclude the possibility of this enzyme being present in small quantities or in a form not disclosed by the method. These qualifications are necessary for any negative result given by histochemical methods.

The sensitivity of the chicken, cat and man to organophosphorous neurotoxicity and the insusceptibility of rodents is one of the more intriguing aspects of this problem. Neither rat nor guinea-pig show paralysis, and in the rat the activity of pseudocholinesterase may be depressed for many weeks without serious consequences (Mendel & Myers, 1952). The anatomical distribution of damaged fibres after tri-*o*-cresyl phosphate poisoning in the chicken and in man would appear to be closely analogous (Cavanagh, 1954 ; Aring, 1942), and it is probable that the damage in the nervous system of the cat may be similar, although there is less precise information about this species (Smith & Lillie, 1931 ; Kidd & Langworthy, 1933). If, as the hypothesis of Davies *et al.* (1960) implies, intoxication by phosphorofluoridates of the neurone or its fibre endings is the simple result of release of fluorine ions interfering with a normal metabolic process, lack of correlation between susceptibility to paralysis and any particular species distribution of cholinesterase would suggest that species variation in response to poisoning may reflect a differing sensitivity of the neurones to the postulated toxic radicle rather than to any other factor. Moreover, distribution of cholinesterases does not offer any explanation for the finding that in the chicken as in man it is the distal portions of the long spinocerebellar and ventral descending tracts of the spinal cord of the chicken that are affected. If mere proximity to centres of high cholinesterase content were important a totally different distribution of lesions would be expected. It would therefore appear that, while cholinesterases may be essential to the toxic process, the distribution of structural damage is likely to be controlled by other factors.

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ACTION OF 5,5-DIETHYL-1,3-OXAZINE-2,4-DIONE (DIOXONE) ON RESPIRATION AND CIRCULATION

BY

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The action of 5,5-diethyl-1,3-oxazine-2,4-dione (dioxone) has been studied in rats, rabbits, cats and dogs. Dioxone produced a marked stimulation of respiration in anaesthetized, decerebrate and spinal animals when given in doses that did not induce convulsions. This effect was generally accompanied by a rise in blood pressure, which was more marked in cats than in dogs and rabbits. Dioxone antagonized the respiratory and circulatory depression due to pentobarbitone. The respiratory stimulating effect of dioxone appears to be 2 or 3 times greater than that of leptazol, and comparable to that of megimide. Like leptazol, nikethamide and megimide, dioxone has no direct effect on cardiac function. Dioxone did not elicit respiratory and blood pressure changes when allowed to come in contact with the carotid sinus receptors. Dioxone enhanced the reflex excitability of bulbar centres, as demonstrated by the increase in respiratory response either to temporary common carotid artery occlusion in dogs or to electrical stimulation of the central cut end of Hering's nerve in the cat. Dioxone also reduced the inhibition of respiration induced by electrical stimulation of the central cut end of the vagus nerve. Whether this central action of dioxone is direct or not cannot at present be elucidated, though a section at intercollicular level did not prevent the respiratory stimulation produced by this substance.

Dioxone (5,5-diethyl-1,3-oxazine-2,4-dione) is a new compound, synthesized by Testa, Fontanella, Cristiani & Gallo (1959), that possesses convulsant properties (Maffii & Silvestrini, 1961) and antagonizes the toxic and lethal effect of barbiturates and other central nervous system depressants.

During the investigation of the convulsant activity of dioxone (Maffii, Dezulian & Silvestrini, 1961), as well as in the study of its protective effect against acute barbiturate intoxication (Maffii & Serralunga, 1961), it was observed that this substance markedly stimulated the rate of respiration. It appeared therefore of interest to determine the effect of dioxone on respiration as well as on the circulation. In the present paper we report the experimental results of such a study that has been carried out on different animal species in comparison with other analeptics.

METHODS

The experiments were done on Wistar rats (180 to 210 g), guinea-pigs, rabbits, cats and dogs. To study the changes in rate of respiration, rats were given pentobarbitone sodium

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by intraperitoneal injection in doses of 50 mg/kg. A group of animals was kept as controls, while other groups were given various subcutaneous doses of dioxone or leptazol 5 min after the administration of the barbiturate. In other experiments, phenobarbitone was used in doses of 120 mg/kg intraperitoneally, and the analeptics were administered 30 min later. The respiratory frequency was measured every 10 min for 2 hr, by direct observation of the animals.

Oxygen consumption. For the oxygen consumption studies, male rats fasted for 15 hr were given pentobarbitone sodium intraperitoneally in a dose of 50 mg/kg, and then put individually into the thermoregulated chamber of a closed-circuit apparatus. The system was filled with air, carbon dioxide being absorbed by passing the air through two bottles containing 30% potassium hydroxide solution and a filter made of quicklime. The oxygen consumption was evaluated through the vol. reduction of the reservoir. The reduction in vol./given time was expressed as dry gas, at normal conditions (0° C and 760 mm Hg), and then reduced to ml. of oxygen/100 g of body weight/min. Each animal received in different stages at 3 days' interval the following treatments: (*a*) pentobarbitone intraperitoneally alone, (*b*) pentobarbitone and 5 min later the analeptic by subcutaneous injection. The cross-over experiment was designed on a Latin square basis. Measurements were always started 10 min after the animal was injected with pentobarbitone so that the oxygen consumption was determined during the period from the 10th to the 30th min after the barbiturate. Animals which awakened or had convulsive attacks during this period because of the effect of the stimulant agents were discarded.

Respiration. The respiratory movements in rabbits, cats and dogs were recorded by 3 different devices: (*a*) Marey tambours connected with the side arm of a tracheal cannula; (*b*) the respirometer of Anderson (1953) connected with a tracheal cannula in order to evaluate tidal air (both with kymograph recordings); (*c*) a pneumometer fixed to the thorax of the animal, and connected with a pressure transducer, the signal of which was amplified by a DC amplifier and recorded on a multichannel electronic (Sanborn) recorder.

Cardiovascular system. Blood pressure of rabbits, cats and dogs was recorded on a kymograph through mercury manometers, as well as on an electronic recorder, through a DC preamplifier, by means of a pressure transducer connected with cannulae inserted either into the femoral or into the carotid artery. Strength of contraction of the dog's heart was recorded by means of a strain gauge arch (Boniface, Brodie & Walton, 1953) fixed to the myocardium of the left ventricle, according to the method described by Cotten & Bay (1956). Operations were performed in animals under pentobarbitone anaesthesia and under artificial respiration provided by a Palmer pump.

Decerebrate and spinal preparations. Decerebration was done under ether anaesthesia by sectioning the mesencephalon at the intercollicular level on the dorsal side and at the level of mammillary bodies on the ventral side. Both common carotid arteries were occluded at the moment of the section. Experiments were started at least 1 hr after the section. In spinal preparations the section at C2 was preceded by the injection of a few drops of 1% procaine solution into the medulla in order to reduce traumatic shock. The excitability of bulbar centres was studied in dogs through the responses elicited by common carotid occlusion and stimulation of the cut central end of the vagus nerve and through the carotid sinus nerve (Hering's nerve) stimulation in cats. Two doses of dioxone were usually administered: one that did not produce appreciable respiratory stimulation, and the other that increased the rate and/or depth of respiration. In dogs under pentobarbitone anaesthesia carotid sinus reflexes were elicited by occlusion of one common carotid for 30 sec. This treatment produces an increase in rate and depth of respiration, a rise of arterial pressure and an increase in heart rate (Heymans & Neil, 1958).

Temporary inhibition of respiration was produced in dogs under pentobarbitone anaesthesia by stimulation of the central end of the vagus nerve sectioned at the neck, with square-wave currents (frequency, 50/sec for 20 sec). Intensities of current were used that were slightly greater than threshold.

Stimulation of the carotid sinus nerve. In cats under pentobarbitone anaesthesia (60 mg/kg intraperitoneally) the carotid sinus nerve was dissected clear of surrounding structures and cut. The central end was stimulated with square-wave stimuli delivered through an electronic stimulator. The duration of stimulation was 30 sec; each single square stimulus had a duration of 0.26 msec. The frequency was varied from 2 to 20 stimuli/sec in order to produce different effects on respiration.

Carotid sinus chemoreceptors. To study the direct effect of dioxone on the carotid sinus chemoreceptors of cats, the lingual and thyroid arteries were cannulated and an injection made alternately in one and then the other artery. Lobeline in doses of 100 and 200 μg was injected to check the response of the preparation.

Isolated guinea-pig auricles were prepared according to the method of Giotti & Nardini (1953).

Solutions. Dioxone was dissolved in distilled water at concentrations from 0.5 to 1%.

RESULTS

Effects on respiratory function

Rats. The effect of dioxone on the frequency of respiration depressed by intraperitoneal pentobarbitone and phenobarbitone was studied by measuring the number of respiratory movements/min every 10 min. Dioxone and leptazol were administered 5 min after the barbiturates.

Fig. 1 shows the stimulation of respiration produced by dioxone in animals given pentobarbitone 50 mg/kg intraperitoneally. Within 60 to 85 min dioxone

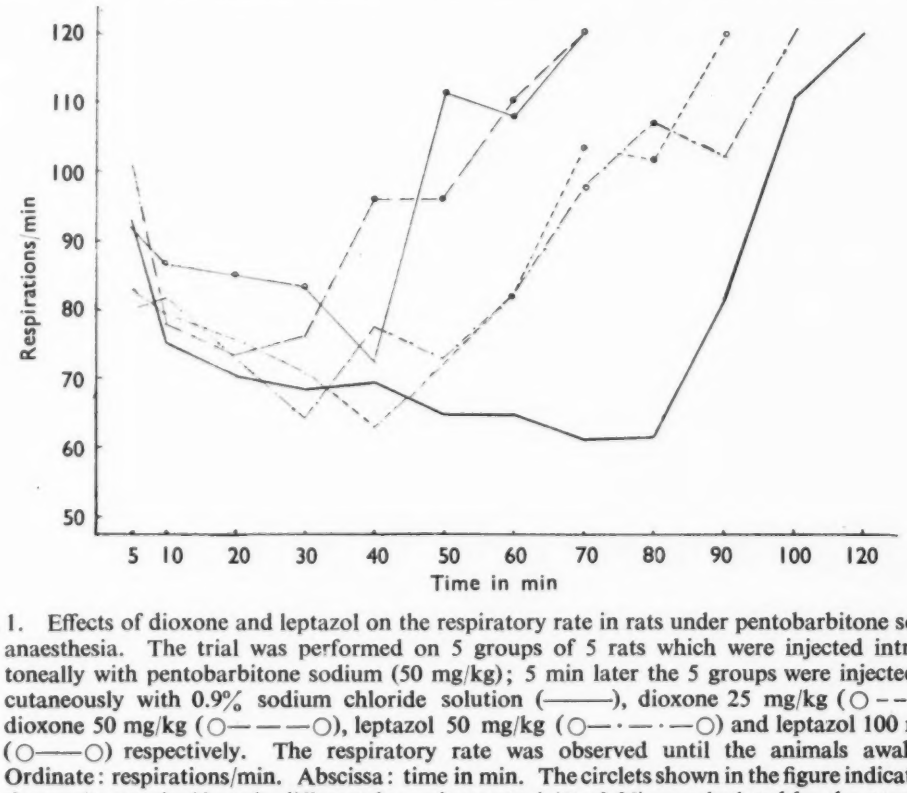

Fig. 1. Effects of dioxone and leptazol on the respiratory rate in rats under pentobarbitone sodium anaesthesia. The trial was performed on 5 groups of 5 rats which were injected intraperitoneally with pentobarbitone sodium (50 mg/kg); 5 min later the 5 groups were injected subcutaneously with 0.9% sodium chloride solution (———), dioxone 25 mg/kg (○ - - - ○), dioxone 50 mg/kg (○— — —○), leptazol 50 mg/kg (○—·—·—○) and leptazol 100 mg/kg (○——○) respectively. The respiratory rate was observed until the animals awakened. Ordinate: respirations/min. Abscissa: time in min. The circlets shown in the figure indicate that the result was significantly different from the control ($P=0.05$) as calculated by the t test.

antagonized almost completely the depressant effect of pentobarbitone in subcutaneous doses of 25 and 50 mg/kg. Leptazol was much less effective, as the doses required were twice as large as those of dioxone. Furthermore, leptazol was found to be completely ineffective at the lower dose studied (25 mg/kg). Dioxone was definitely effective at a dosage of 25 mg/kg, although its action was brief. At a dose of 50 mg/kg the activity of dioxone was greater and was exerted for at least 1 hr. Leptazol was completely inactive at a dose of 25 mg/kg, and in doses of 100 mg/kg it appeared less effective both in intensity and duration than dioxone at 50 mg/kg. It is obvious that in the case of phenobarbitone the depression lasts longer than the effect of dioxone, and the general picture of the results is quite different from that obtained in animals given pentobarbitone, the duration of action of which is about equal to that of dioxone.

In order to ascertain whether the stimulating effect of dioxone on respiration results in an increase in oxygen consumption, experiments were carried out on rats. Animals given pentobarbitone in doses of 50 mg/kg intraperitoneally showed a reduction of 50% in the oxygen consumed/min/100 g body weight. As shown in Table 1, dioxone (25 and 50 mg/kg) restored to some extent the oxygen consumption which had been depressed by pentobarbitone. Leptazol, tested for the same activity under the same conditions, appeared obviously less active than dioxone.

TABLE 1

OXYGEN CONSUMPTION OF RATS AFTER PENTOBARBITONE AND ANALEPTIC AGENTS

Observations were made over a 20 min period. Pentobarbitone was injected intraperitoneally (i.p.) and dioxone and leptazol subcutaneously (s.c.)

Treatment	Dose mg/kg	No. of determinations	ml. oxygen/min/100 g body weight	s.e.	% changes
Controls (awake)	—	12	3·19	0·08	—
Pentobarbitone	50 i.p.	4	1·55	0·07	—
Pentobarbitone + dioxone	50 i.p. + 25 s.c.	4	1·89	0·10	21·93
Pentobarbitone	50 i.p.	4	1·57	0·06	—
Pentobarbitone + dioxone	50 i.p. + 50 s.c.	4	2·41	0·07	53·51
Pentobarbitone	50 i.p.	4	1·79	0·07	—
Pentobarbitone + leptazol	50 i.p. + 100 s.c.	4	2·68	0·35	49·72

Rabbits. In animals anaesthetized with urethane (1 mg/kg intraperitoneally), dioxone in doses ranging from 3 to 5 mg/kg injected intravenously produced an increase in the respiratory amplitude in 2 out of 4 animals, and an increase in the respiratory frequency in 3 out of 4 animals, for a period of 12 to 20 min. Leptazol produced similar effects at a dose of 10 mg/kg.

A second group of animals was anaesthetized with pentobarbitone injected intravenously in doses of 20 and 30 mg/kg, which produced obvious depression of both the frequency and amplitude of respiration ; 10 and 20 mg/kg of dioxone appreciably

increased the frequency and amplitude of respiration, and similar effects were obtained with 30 mg/kg of leptazol.

In animals treated with morphine 10 mg/kg intravenously, dioxone in doses as low as 4 mg/kg stimulated respiration, but also produced marked signs of motor excitation, and even clonic convulsions in some cases. Electroencephalographic registrations, performed simultaneously, demonstrated that the convulsant effects of

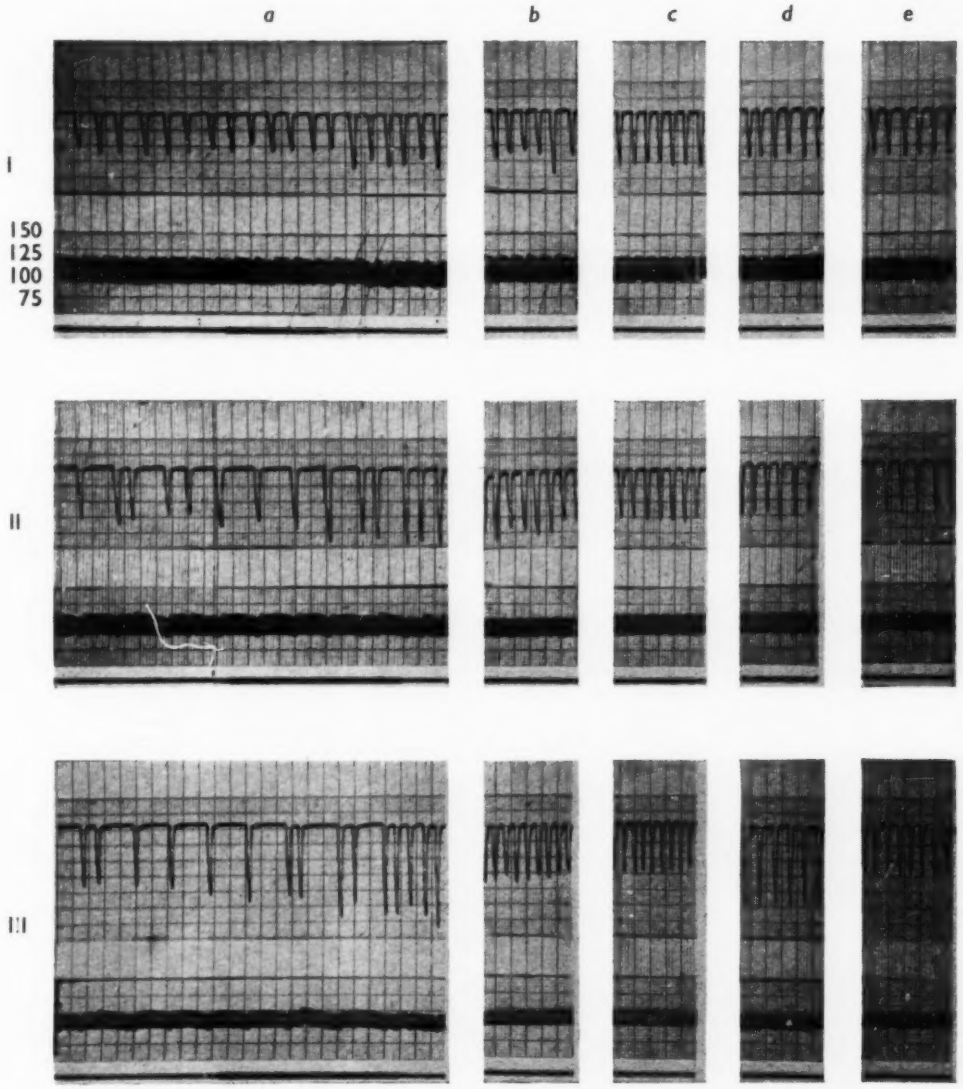

Fig. 2. Dog anaesthetized with pentobarbitone sodium (40 mg/kg intravenously). Records from above downwards: pneumogram recorded by pressure transducer through thoracic pneumograph (inspiration downwards); carotid blood pressure recorded by a pressure transducer and signal/time marker (1 sec). The effects of dioxone injected intravenously: I, 2 mg/kg 15 min after pentobarbitone; II, 4 mg/kg 15 min after a further 15 mg/kg of pentobarbitone; III, 8 mg/kg 15 min after a further 10 mg/kg of pentobarbitone. The intervals between the tracings horizontally were 5 min. There was a 40 min interval between I and II and between II and III.

dioxone were greatly increased in animals pretreated with morphine ; this has also been observed following leptazol 10 mg/kg. Existing data show that there is synergism between morphine and leptazol in the production of convulsions (Hazleton & Koppanyi, 1941).

Cats. The experiments were performed on animals anaesthetized with chloralose (70 mg/kg intraperitoneally), urethane (1 g/kg intraperitoneally) and pentobarbitone (60 mg/kg intraperitoneally). In chloralosed animals 2.5 mg/kg of dioxone produced a clear respiratory stimulation, though diffuse muscular clonus was also observed. In animals treated with urethane, higher doses, namely, 5 to 10 mg/kg, were necessary and produced essentially an increase in amplitude. In this case the convulsive phenomena occurred only at much higher doses. In cats given pentobarbitone the effects of dioxone were found to be dependent on the depth of anaesthesia and the depression of the respiratory function ; stimulation was obtained with 4 to 8 mg/kg.

Dogs. In animals deeply anaesthetized with 60 mg/kg of pentobarbitone injected intraperitoneally, dioxone caused a moderate increase of the respiratory frequency and amplitude in intravenous doses as low as 2 mg/kg (Fig. 2). With 6.8 and 10 mg/kg or more the effect was more obvious and lasted for 15 to 30 min (Fig. 3).

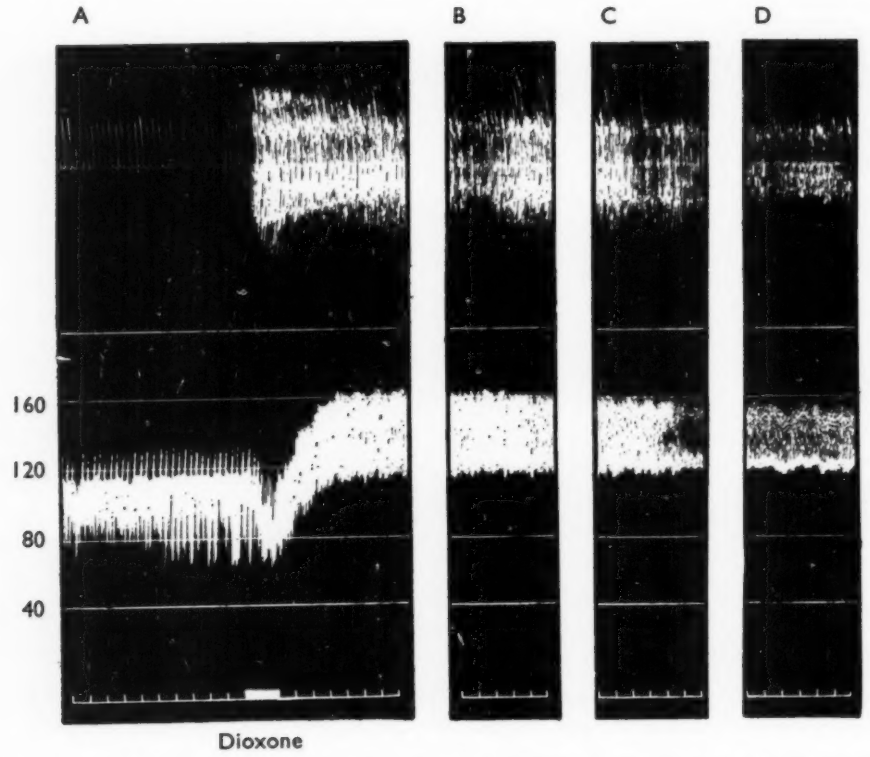

Fig. 3. Effect of a high dose of dioxone on respiration and blood pressure of dog under pentobarbitone sodium 40 mg/kg injected intravenously. A: At the signal dioxone 15 mg/kg was injected intravenously. The interval between A and B was 5 min, between B and C 10 min, and between C and D 30 min. Time marker, 1 min.

In general, the frequency and amplitude were found to be increased to the same extent. The actions of dioxone and megimide on the respiration of pentobarbitone-depressed dogs were found to be equivalent.

Effect of dioxone on decerebrate animals

Decerebrate rabbits. Dioxone in intravenous doses of 2.5 to 7.5 mg/kg produced stimulation of respiration in 7 out of 8 decerebrate rabbits. In some cases there was, a few sec after the injection, an increase in amplitude, and in other animals an increase of frequency that was usually accompanied by increase in rigidity and slight motor stimulation.

Decerebrate cats. The activity of dioxone given in doses of 2.5 to 5 mg/kg to 8 decerebrate cats appeared greater than in decerebrate rabbits, and the increase in the rate of respiration was often accompanied by increase in the amplitude of single movements, as demonstrated by the increase in tidal air. Thus the effect of dioxone in decerebrate cats appears to be similar, or even greater, than in deeply anaesthetized animals.

Leptazol has little or no stimulating effect on respiration of decerebrate animals, even when given in doses twice those of dioxone. Megimide, on the contrary, is still effective in decerebrate animals in doses similar to dioxone.

Decerebrate dogs. Dioxone 5 to 7 mg/kg caused a slight augmentation of respiration in 4 out of 6 decerebrate dogs. In 2 animals receiving 2.5 mg/kg, dioxone appeared ineffective. Contrary to what occurred in cats, mesencephalic section of the brain stem appeared to reduce the respiratory stimulation produced by dioxone.

Effects of dioxone on blood pressure

The experiments were carried out on rabbits, cats and dogs, both anaesthetized and decerebrate. In the different species the common feature of the action of dioxone may be summarized as follows. In doses from 2 to 10 mg/kg intravenously dioxone produced generally an increase in blood pressure, though this action was not as constant as the effect on respiration, and depended upon the pressure level before treatment, being small or absent in lightly anaesthetized animals, with an initial systolic pressure of 110 to 140 mm of mercury. In deeply anaesthetized animals with an initial blood pressure of less than 100 mm of mercury, dioxone (3 to 10 mg/kg) produced a 15 to 40% increase in the pressure level. With higher doses, when muscular twitches or clonic convulsions were evident, there was always an increase in blood pressure. The rise in blood pressure was generally prolonged, though when hypotension was marked, due to high doses of a barbiturate, the effect of dioxone (5 to 10 mg/kg intravenously) usually lasted for 3 to 6 min, and further administration was necessary in order to maintain satisfactory levels. A brief analysis of the results follows.

Rabbits. The blood pressure level was registered in unanaesthetized and curarized animals, in animals treated with morphine, as well as in spinal and decerebrate animals. Respiration and, in some cases, electroencephalographic records were registered at the same time as the haemodynamogram. In unanaesthetized rabbits,

dioxone in a dose of 5 mg/kg intravenously produced a rise in blood pressure of 40 to 80% and at the same time convulsive phenomena in the electroencephalogram at cortical level, and marked respiratory stimulation. Greater effects were usually seen after doses of 8 to 10 mg/kg. Both bemegride and leptazol exert similar effects, the former in the same dose range as dioxone, and the latter in doses of 15 to 20 mg/kg.

When repeated doses of dioxone were administered at intervals allowing a reduction of 50% of the hypertensive response, the rise in blood pressure reappeared after each injection until the fourth or fifth one, after which further administration was unable to produce changes in the blood pressure. Doses of 5 and 10 mg/kg intravenously were repeated 10 times at intervals, respectively of 10 and 15 min in 3 rabbits each, without causing death even when severe convulsions were present.

In curarized rabbits (tubocurarine 0.5 mg/kg intramuscularly) the effects of dioxone did not substantially differ from those described for unanaesthetized animals. In animals given morphine (10 mg/kg intravenously) lower doses (2 to 4 mg/kg) of dioxone elicited the hypertensive as well as the convulsive response, together with respiratory stimulation. In decerebrate rabbits dioxone produced only slight increase in blood pressure in 2 out of 5 animals, and in 2 cases the

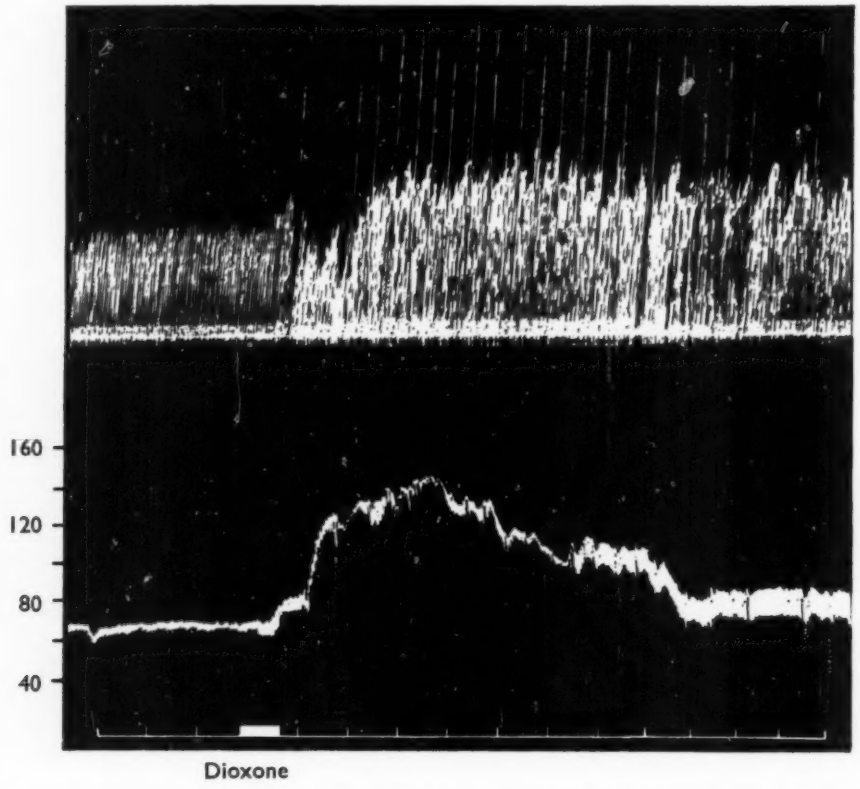

Fig. 4. Effects of intravenous dioxone (5 mg/kg) on the decerebrate cat. Records from above downward: tidal air recorded through an Anderson respirometer, carotid blood pressure recorded by mercury manometer and signal/time marker (1 min).

stimulation of respiration caused by doses of 5 and 7.5 mg/kg intravenously was accompanied by some lowering of blood pressure.

Cats. In curarized animals (tubocurarine 0.6 mg/kg intramuscularly) dioxone produced transitory hypertension in doses (5 to 10 mg/kg intravenously) which caused convulsions, as demonstrated by electroencephalographic recordings. In cats anaesthetized with pentobarbitone sodium (60 mg/kg intraperitoneally) dioxone produced a rise in blood pressure even in a dose of 2 mg/kg intravenously. This effect lasted usually for 15 to 20 min and was not always accompanied by respiratory stimulation. With higher doses the hypertensive effect was not more marked, but it was accompanied by an increase in rate and/or amplitude of respiration, and with doses higher than 8 to 12 mg/kg by convulsions. In decerebrate cats dioxone still produced a rise in blood pressure (Fig. 4) similar to that observed in anaesthetized ones. In spinal cats, dioxone (2 to 10 mg/kg) had very inconsistent effects on blood pressure, and produced in 4 out of 6 animals a lowering which disappeared either after sectioning both vagi or after atropine 0.5 mg/kg intravenously.

Dogs. In animals in deep pentobarbitone anaesthesia (40 mg/kg intravenously) dioxone produced a smaller rise in blood pressure than that seen in cats. The rise seemed to be directly related to the degree of respiratory stimulation. In decerebrate dogs dioxone had approximately the same slight effects as observed in anaesthetized animals.

Effects of dioxone on the heart

Dogs. The effects of dioxone on cardiac contractile force and electrocardiogram were studied in dogs under deep pentobarbitone anaesthesia (40 mg/kg intravenously in repeated doses) and under artificial respiration. Dioxone in doses of 1 to 3 mg/kg had no appreciable effect. Higher doses (9 to 18 mg/kg) slightly raised the blood pressure, but had no effect on the heart. Only in particularly depressed animals was the rise in blood pressure produced by dioxone secondarily followed by slightly increased contractions. In some experiments a comparative study was carried out with leptazol, megimide and nikethamide, the doses being adjusted so that leptazol was administered in the same range as dioxone, leptazol from 10 to 30 mg/kg and nikethamide from 10 to 40 mg/kg. Direct comparisons in the same animal demonstrated that the three substances had no significant action on the heart.

Isolated guinea-pig auricles. Nikethamide, megimide, leptazol and dioxone, given in concentrations of 1 to 20 μg/ml., had no inotropic or chromotropic effect on this preparation.

Effects of dioxone on the excitability of bulbar centres

Response to common carotid occlusion. It is known that occlusion of the common carotid artery for 30 sec causes a rise in blood pressure and an increase in rate and depth of respiration (Heymans & Neil, 1958). When dioxone was given in doses of 2 mg/kg intravenously, which in the dog produced little stimulation of respiration, the increase in rate and depth of respiration induced by common carotid occlusion appeared to be potentiated for some time, though there was

little or no effect on the pressure response. Following 4 mg/kg intravenously the respiratory rate was increased by dioxone itself, and the changes in the respiratory response to common carotid occlusion became difficult to evaluate.

Response to central stimulation of the vagus in the dog. The temporary inhibition of respiration produced by electrical stimulation of the central end of the vagus was slightly reduced in dogs given dioxone at 2 mg/kg intravenously. Higher doses (4 mg/kg intravenously), which produced some stimulation of respiration, usually abolished for some 10 min the inhibition of respiration caused by stimulation of the vagus.

Response to central stimulation of Hering's nerve in the cat. Electrical stimulation of the central end of Hering's nerve of the cat induces different effects according to the frequency of stimulation. As demonstrated by Douglas & Schaumann (1956), by varying the frequency from 2 to 40 stimuli/sec, it is possible to elicit a respiratory response and a pressure rise that become clear at 4 or 6 stimuli/sec and are then greater in proportion to the frequency of stimulation. In 4 cats anaesthetized with pentobarbitone, the effects of stimulation of the central end of Hering's nerve at various frequencies were studied before and after the administration of dioxone in doses that did not affect respiration. As shown in Fig. 5, under the effect of dioxone the threshold of frequency of stimulation needed to elicit a minimal response of respiration was lowered and the effect of supraliminal stimulation enhanced. When doses of dioxone were given high enough to produce an increase in rate and depth of respiration (4 mg/kg intravenously), the response to Hering's nerve stimu-

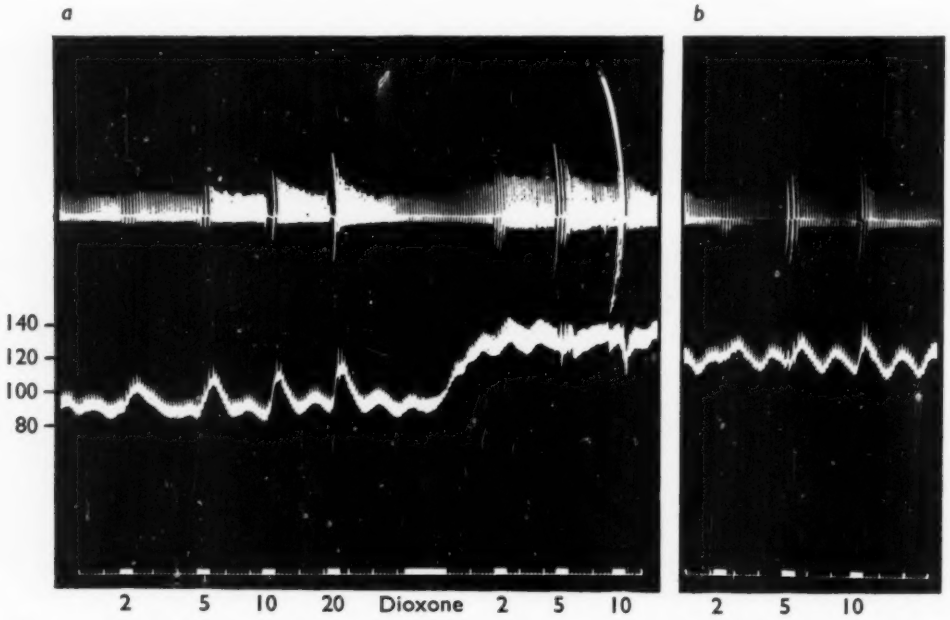

Fig. 5. Effects of dioxone on the respiratory and blood pressure response to the stimulation of Hering's nerve in the anaesthetized cat. Signal/time marker (1 min). After stimulation with frequencies of 2, 5, 10, and 20/sec, dioxone, 4 mg/kg, was injected intravenously. Then the stimulation was repeated at 2, 5, and 10/sec. Interval between A and B was 60 min.

lation appeared very marked. However, the hypertensive response was not changed after both dose levels of dioxone.

Lack of peripheral action of dioxone on carotid sinus

In order to exclude a possible action of dioxone on respiration and blood pressure by a peripheral mechanism, for example, by stimulating the receptors of glomus caroticus, experiments were performed on 4 cats by injecting the drug in the lingual and in the thyroid artery: in the first case the drugs pass through the carotid bifurcation, and in the second not. Dioxone in doses of 2.5 mg/kg did not act differently whether or not it came in contact with the carotid glomus. Lobeline in a dose of 100 and 200 μg produced in the same animals a clear stimulation of respiration only when injected in the thyroid artery.

Two dogs whose bifurcation of carotids was perfused for 15 min with Ringer containing a concentration of dioxone as high as 1% did not show any increase in respiration. Lobeline at a concentration of 50 μg/ml. produced a definite effect after a few sec.

DISCUSSION

The results show that dioxone produces a marked stimulation of respiration. This action is very clear in animals depressed by barbiturates or other anaesthetics and may be observed even with doses of 2 mg/kg intravenously. Dioxone produces changes both in frequency and amplitude of respiration and is able to restore to some extent the oxygen consumption depressed by pentobarbitone. The effect on respiration is still present in decerebrate cats and rabbits but is greatly decreased in decerebrate dogs. Together with the increase in respiration, dioxone produces a rise in blood pressure that is usually inversely proportional to the pretreatment levels.

This rise in blood pressure, however, is less evident in rabbits and dogs. In fact, in those animals greater doses must be employed to cause a rise in blood pressure than are necessary to produce augmentation of respiration. The hypertensive effect of dioxone is not modified by decerebration in cats, but is variable in decerebrate dogs and rabbits.

In spinal cats dioxone tends to lower the blood pressure, and this action is inhibited either by section of both vagi or by atropinization. This finding suggests that the stimulation produced by dioxone on bulbar centres results in generalized discharges involving the main efferent paths, and that the hypertension observed in intact animals is due to the prevalence of sympathetic over vagal stimulation. Submedullary sections, by abolishing the sympathetic component, allow the vagal stimulation to appear. This hypothesis is also in agreement with the finding of brief episodes of hypotension occasionally observed in some animals immediately after, or during, the intravenous injection of dioxone and rapidly followed by the usual rise in blood pressure.

As far as the mechanism of action is concerned, it can be stated that the effects of dioxone on respiration and blood pressure are due to its action on the central nervous system. By studying the reflex excitability of the bulbar centres it has

been demonstrated that dioxone, in doses that do not affect respiration, or stimulate it very little, can produce an increase in the respiratory reflex stimulation due to a rapid hypotension at the level of carotid glomus, and also an augmentation of the reflex response due to electric stimulation of Hering's nerve.

On the other hand, the period of apnoea which may be produced in dogs by stimulation of the central portion of the sectioned vagus nerve may be reduced or abolished for a short time following the administration of dioxone. The hypertensive response to common carotid occlusion is usually unmodified or little increased. It seems likely, on the basis of these findings, that dioxone acts on centres that regulate respiration both by increasing the excitability of these structures to reflex stimulation and by insulating them from inhibitory effects. Whether this action of dioxone is direct or mediated through the effect on other parts of the central nervous system requires further elucidation. However, the results obtained in decerebrate animals demonstrate that the connexion of the bulbar centres with structures lying at higher than mesencephalic levels is not strictly necessary for this action of dioxone. This fact, which has been clearly established in cats, is not so definite in rabbits and dogs.

In dogs, respiratory centres are more susceptible than vasomotor centres to stimulation by dioxone, lower doses being necessary to elicit increase in respiration than to produce a rise in blood pressure.

In cats the pattern of the action of dioxone differs substantially because the hypertensive effect of the drug may sometimes be demonstrated in the absence of respiratory stimulation. However, in these conditions a lowering of the threshold for reflex stimulation of the respiratory mechanism through the stimulation of Hering's nerve can be demonstrated. On the basis of studies by Kissel & Domino (1959) on the influence of blood pressure changes on the reflex excitability of the central nervous system, we can exclude the possibility that the rise in blood pressure caused by dioxone in these circumstances is responsible for the increase in the excitability of respiratory centres.

Our experiments have confirmed the lack of any direct influence of leptazol and nikethamide on cardiac function, in agreement with the findings of Goodman & Gilman (1955) and Krop (1944). Moreover, megimide and dioxone have also no significant effect on the heart. This emphasizes the limits of the practical use of analeptic drugs, though within these limits it would appear that dioxone has pharmacological properties of interest in therapeutics.

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FATE OF SYNTHETIC OXYTOCIN ANALOGUES IN THE RAT

BY

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The disappearance of phe^3-oxytocin, val^3-oxytocin and oxytocin from the circulation of male rats was shown to be due to qualitatively similar mechanisms, that is, it depends on uptake in the kidneys and organs of the splanchnic vascular area. However, compared to phe^3-oxytocin and oxytocin (whose half-lives were essentially similar) val^3-oxytocin took twice as long to reach half its initial blood concentration. In lactating rats the mammary glands probably participated in the uptake of phe^3-oxytocin, but the rate of disappearance of val^3-oxytocin was not different from that in non-lactating animals. In male nephrectomized rats without splanchnic circulation, phe^3-oxytocin, unlike val^3-oxytocin, was quickly distributed in a volume equal to two-thirds of the total body water. Using oxytocin as the standard, val^3-oxytocin and phe^3-oxytocin were more potent when assayed on a superfused uterus or on a rat uterus *in vivo* than when assayed by the pharmacopoeial method (1958) on the isolated uterus in an organ bath. The difficulties of assaying oxytocin analogues against oxytocin (or the international standard preparation) are discussed.

In a previous investigation (Ginsburg & Smith, 1959) we have shown that, after intravenous injection into rats, oxytocin disappeared rapidly from the circulation, this being due mainly to preferential uptake by the kidneys, organs of the splanchnic vascular area and the lactating mammary gland.

Recently two synthetic analogues of oxytocin, phenylalanyl^3-oxytocin (phe^3-oxytocin) and valyl^3-oxytocin (val^3-oxytocin) were made available to us through the generosity of Sandoz & Co., Basle. The effect of val^3-oxytocin on the uterus *in situ* in rats (Boissonnas, Guttmann, Jaquenoud, Waller, Konzett & Berde, 1956), cats (Berde, Doepfner & Konzett, 1957) and women (Smyth, 1958) is greater than would be expected from the potency estimated *in vitro*, and the present investigation was undertaken in the first place to determine whether some difference in the fate of val^3-oxytocin in the body would account for this apparently enhanced activity *in vitro*. Arising from this investigation, some experiments are described on the relative potency of oxytocin and the oxytocin analogues on rat uteri under different conditions.

METHODS

Adult albino rats of both sexes weighing 180 to 250 g were used.

Assays of oxytocic activity

Rat uterus in vitro. Oxytocins in plasma extracts were assayed on superfused uteri of rats in oestrus or pro-oestrus. The constant temperature of the superfusing fluid was within

the range 30 to 33° C depending upon the reactivity of the uterus; the rate of flow was 3 to 4 ml./min and the composition of the superfusion fluid was that given by Ginsburg & Smith (1959). In some experiments assays were carried out on a uterus bathed in 2 ml. of the rat uterus solution described in the British Pharmacopoeia, 1958.

Rat uterus in vivo. The method of Bell & Robson (1937) for recording uterine movements *in situ* in guinea-pigs was applied to rats. Rats in pro-oestrus or oestrus were anaesthetized with 0.8 ml. of a 25% solution of urethane per 100 g body weight injected subcutaneously. The left horn was isolated from other abdominal viscera and covered with a flattened glass filter funnel; a thread tied in a loose loop around the middle of the uterus was passed through the spout of the funnel and attached to a frontal writing lever. The vaginal end of the uterus was fixed in position with a pin and the ovarian end fixed by a thread passed through a small hole in the funnel. Injections were given into a cannulated vein.

Extraction of oxytocin analogues from plasma

The method described in a previous paper (Ginsburg & Smith, 1959) for the extraction of oxytocin from plasma was used. The mean recoveries of oxytocin analogues added to rat plasma were $83 \pm 2.9\%$ (5) (mean and standard error) for val^3-oxytocin and $96 \pm 7.4\%$ (5) for phe^3-oxytocin.

Standardization of the synthetic oxytocin analogues

1 ml. of the solution of phe^3-oxytocin provided by Sandoz & Co. was stated to possess a vasopressor potency of 1 i.u.; no oxytocic potency was given. Phe^3-oxytocin was therefore assayed on a rat uterus in a 2 ml. organ bath according to the method described in the British Pharmacopoeia (1958). Oxytocin (Pitocin, Parke, Davis & Co.) was used as standard, and doses which gave contractions between 30 and 70% of the maximum were applied. The potency thus estimated was 3.7 ± 0.3 units/ml. (fiducial range at $P = 0.05$). This result was used to express all the doses of phe^3-oxytocin in units of oxytocic activity.

The oxytocic potency of the val^3-oxytocin solution was given as 2.0 i.u./ml. The result of assays using rat uteri in an organ bath was 2.06 ± 0.05 ($P = 0.05$), agreeing with the stated potency.

Estimation of tritium (^3H_2O) in plasma

0.25 ml./100 g body weight of 0.9% sodium chloride solution containing 10 μc/ml. of tritium as tritium oxide was injected intravenously into rats anaesthetized with ether. Sixty minutes after the injection blood was collected from a cannulated carotid artery. Plasma proteins were precipitated by adding to each ml. of plasma 0.93 ml. of 6% trichloracetic acid, so making the ^3H_2O concentration in the supernatant half of that in the original plasma, on the assumption that the water content of plasma was 93%. The supernatant was blended with 2:5-diphenyloxazole in a toluene-ethanol mixture and the radioactivity then measured in a liquid scintillation counter (Ekco type N 612). The ^3H_2O–H_2O mixture used for the preparation of standards also contained 3% trichloracetic acid.

Paper chromatography of oxytocins

The conditions described by Heller & Lederis (1958) for ascending chromatography of oxytocin on paper in an n-butanol:acetic acid:water system were used. The staining method of Reindel & Hoppe (1954) was used to locate the peptides.

RESULTS

Disappearance of injected oxytocins from the circulation

The plan of the experiments followed that described previously for oxytocin (Ginsburg & Smith, 1959): the oxytocin analogue, 200 m-u./100 g, was injected

intravenously into rats anaesthetized with ether. Blood was withdrawn from a carotid artery at intervals, up to 30 min after injection. After extraction from plasma the oxytocin was assayed on the superfused rat uterus. In a preliminary experiment it was found that the rate of disappearance of synthetic oxytocin (Syntocinon, Sandoz) was not different from that for natural oxytocin. It may be concluded that the inert constituents, which in the synthetic preparation are likely to be different from those found in the glandular extracts, do not affect the rate of disappearance of oxytocin from the circulation. It is assumed that inert impurities known to be present in solutions of the oxytocin analogues (Konzett, personal communication) would also be unlikely to affect experimentally determined half-lives.

Fig. 1 shows the half-lives calculated from the rates of disappearance of injected oxytocin and the two synthetic analogues in lactating rats 3 to 14 days post-partum, in intact males, in males without splanchnic circulation (this being arrested by ligation of the coeliac and superior mesenteric arteries and division of the ascending colon and left colic artery between ligatures and ligation of the portal vein) and in male nephrectomized rats. Bilateral nephrectomy significantly slowed the rate of disappearance of all the peptides, the mean half-lives being 1.8, 2.1 and 1.7 times

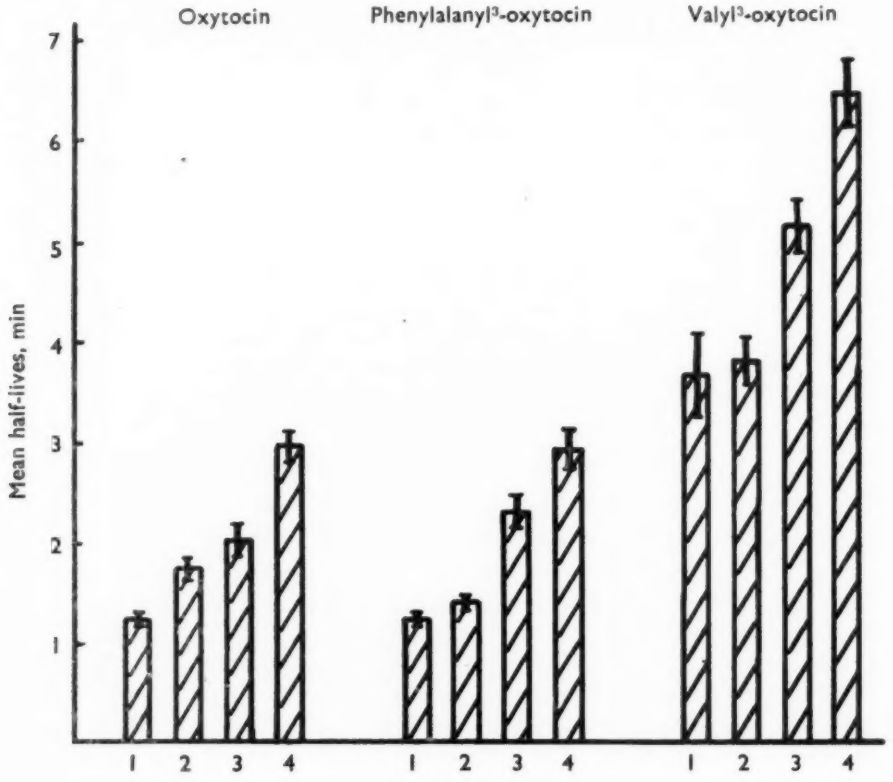

Fig. 1. Half-lives of oxytocin, phe³-oxytocin and val³-oxytocin determined in rats. 1, lactating rats, 3 to 14 days post-partum; 2, intact male rats; 3, male rats without splanchnic circulation; 4, male nephrectomized rats. Each value is the mean of from 5 to 7 experiments ± the standard deviation.

that in intact animals for oxytocin, phe^3-oxytocin and val^3-oxytocin respectively. Arrest of the splanchnic circulation retarded the disappearance of both oxytocin analogues from the circulation, and, in contrast with the results obtained with oxytocin shown in Fig. 1 (see also Ginsburg & Smith, 1958), this retardation was significant at the 5% level of probability. In lactating animals the half-lives of oxytocin and phe^3-oxytocin were significantly shorter than in intact males ($P<0.001$), but the half-life of val^3-oxytocin was unchanged ($P>0.8$). In intact rats, rats with no splanchnic circulation, nephrectomized rats or lactating rats, the mean half-life of phe^3-oxytocin was not significantly different from that of oxytocin. Val^3-oxytocin persisted in the circulation longer than the other peptides, the half-life being approximately double that of oxytocin or phe^3-oxytocin in rats in each of the states investigated.

When oxytocin is injected into a nephrectomized rat without splanchnic circulation, the oxytocin concentration falls for about 10 min when a steady level is reached (Ginsburg & Smith, 1959). Fig. 2B shows the tendency for the concentration of phe^3-oxytocin to reach a steady level at about the same time; in only one experiment out of five, the concentration of phe^3-oxytocin in plasma continued to fall up to 25 min after injection. Thus the preferential uptake of oxytocin and phe^3-oxytocin from the circulation appears to be restricted to the kidneys, the organs of the splanchnic vascular area and the mammary gland. The disappearance of val^3-oxytocin in nephrectomized rats without splanchnic circulation was so slow

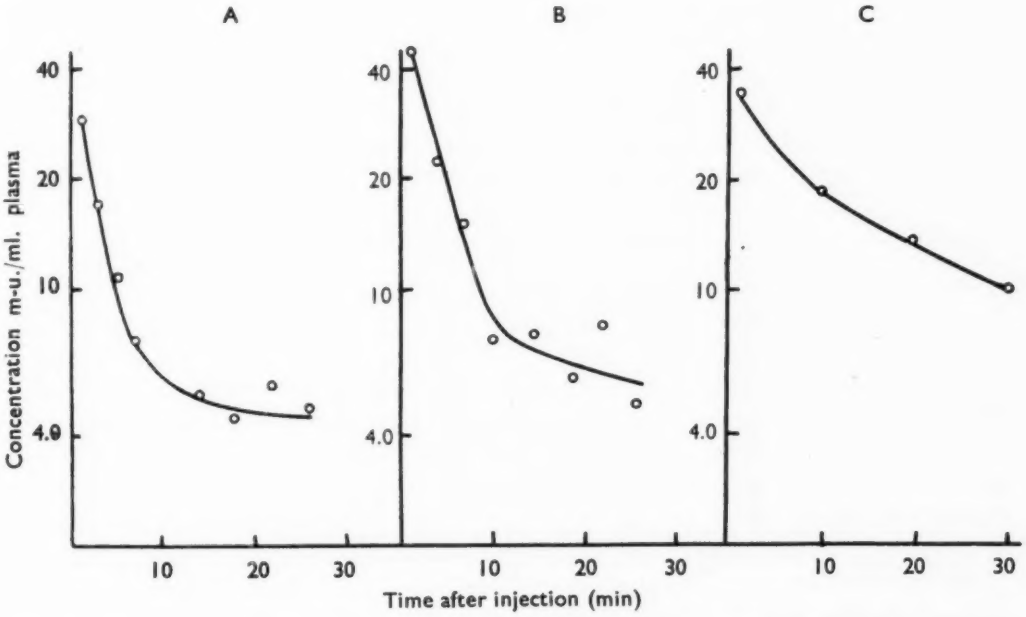

Fig. 2. Plasma concentrations of oxytocin, phe^3-oxytocin and val^3-oxytocin following intravenous injection into male nephrectomized rats without splanchnic circulation. A=oxytocin; B=phe^3-oxytocin; C=val^3-oxytocin. Each point is the mean of from 3 to 5 results.

that even 30 min after injection the mean plasma concentration was over 10 m-u./ml., that is, more than double the equilibrium concentration for the other two peptides (see Fig. 2C).

Tritium oxide space in nephrectomized rats without splanchnic circulation

The equilibrium concentrations of oxytocin and phe^3-oxytocin found in plasma in nephrectomized rats without splanchnic circulation gave volumes of distribution of the order of 40 ml./100 g body weight. The inulin space determined under the same conditions was 12 ml./100 g body weight (Ginsburg & Smith, 1959). For comparison, the tritium oxide space in nephrectomized rats without splanchnic circulation was determined. In four experiments the mean tritium oxide space was 62 ml./100 g original body weight.

Duration of action of oxytocins on the uterus in vivo

If the duration of action of an oxytocin *in vivo* is related to its persistence in the circulation, the action of val^3-oxytocin should be more prolonged than that of the other two peptides. Doses of oxytocin and a synthetic analogue were injected which, judging from preliminary experiments, were expected to be approximately equipotent, that is, to produce similar increases in uterine tonus. The duration of action, taken from the time of injection until the amplitude of the uterine movements returned to the pre-injection level, was compared in 10 pairs of matched injections of oxytocin and val^3-oxytocin. Precise prediction of equipotent doses was not possible; oxytocin produced an excursion of 32.5 mm while the chosen dose of val^3-oxytocin produced an excursion of 30.5 mm (each value is the mean of 8 responses). For each pair of injections the ratio, duration of action of val^3-oxytocin:duration of action of oxytocin, was calculated. The mean ratio for the 10 pairs of injections was 1.4 ± 0.05. Thus, in spite of the slightly greater mean response to oxytocin, the mean duration of action of val^3-oxytocin was significantly longer. In 7 similar experiments the ratio, duration of action of phe^3-oxytocin: duration of action of oxytocin, was 1.0 ± 0.06.

Potency of oxytocin and oxytocin analogues in vivo *and* in vitro

The design of the experiments in which the duration of action of the oxytocins *in vivo* was determined also permitted estimation of their relative potencies *in situ*, increase in tone being used as a parameter for these assays. In four such assays the mean potency of val^3-oxytocin was 3.4 ± 1.5 (fiducial range, $P = 0.05$) times that of oxytocin. In three experiments with phe^3-oxytocin the mean potency was 12.5 ± 4.9 ($P = 0.05$) times greater than that of oxytocin. Fig. 3 gives an example of one of these experiments. The doses of the oxytocin analogues are expressed in units, according to the assays on isolated rat uteri under the conditions given in the British Pharmacopoeia, 1958, in which there was no internal evidence that the assays from which the potencies of the solutions of synthetic oxytocin analogues were originally obtained were invalid. However, because the doses used were restricted to a narrow range (the ratio high dose/low dose was 1.5), deviation from parallelism might have passed unnoticed. In view of the results obtained on rat

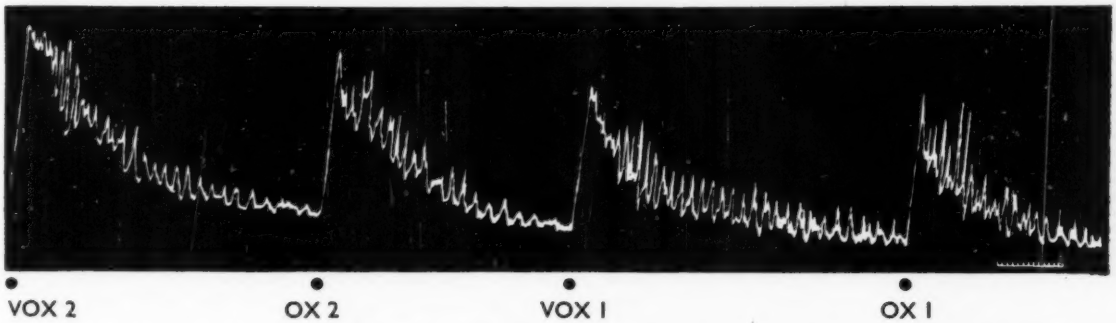

Fig. 3. Assay of val³-oxytocin against oxytocin on an oestrous rat uterus *in situ*. OX 2 and OX 1 are the high and low doses of oxytocin; 200 m-u. and 100 m-u. respectively. VOX 2 and VOX 1 are the high and low doses of val³-oxytocin; 50 m-u. and 25 m-u. respectively. Time, 30 sec.

uteri *in situ*, the potencies of oxytocin and the synthetic analogues were also compared by experiments on the rat uterus *in vitro*.

In each experiment an analogue was compared with oxytocin on an isolated rat uterus upon which 2 assays were performed. In one assay doses were used which gave responses of *less than* 30% of the maximum; in the other the responses were *greater than* 70% of the maximum. The results are shown in Fig. 4. With phe³-oxytocin the estimated potencies at both high and low dose levels agreed with each

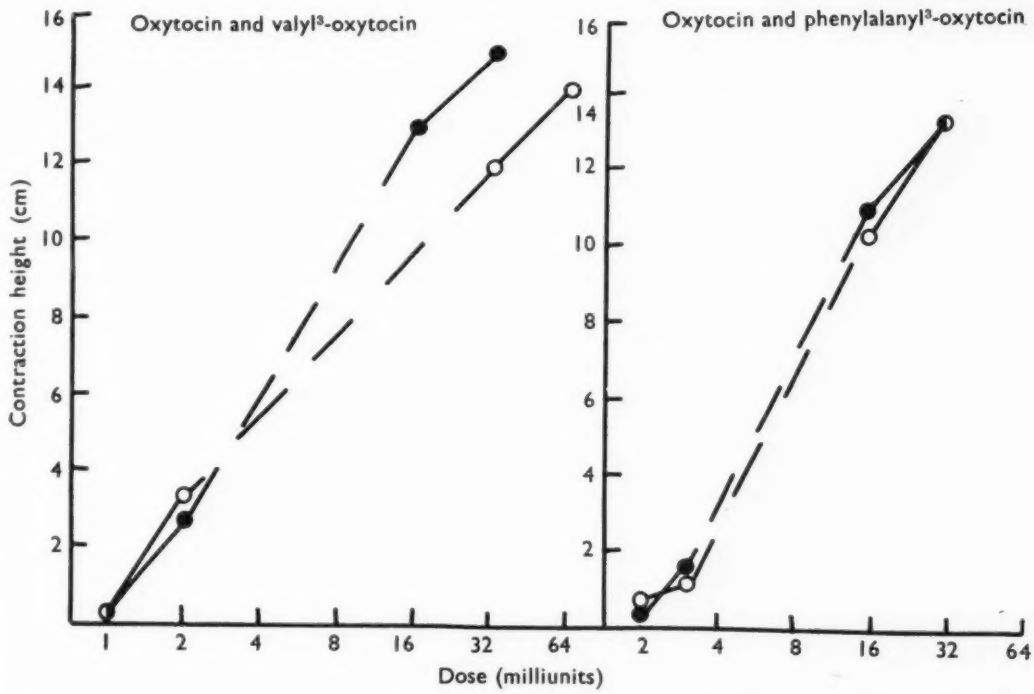

Fig. 4. Log dose-response curves for oxytocin, phe³-oxytocin and val³-oxytocin determined on the isolated rat uterus in an organ bath. ●——●=oxytocin; ○——○=synthetic oxytocin analogue.

other (3.78 and 3.67 i.u./ml. respectively). With val^3-oxytocin the assay using responses below 30% of the maximum agreed well with the potency estimate given by responses between 30% and 70% of the maximum (2.15 i.u./ml. compared with 2.06 i.u./ml.), but at the higher dose level (>70%) the estimate of the potency was 0.96 i.u./ml. Although deviation from parallelism of the log dose-response lines for oxytocin and val^3-oxytocin is not marked enough to be statistically significant over a small range of doses, the potency estimates may differ by a factor of 2, when assays at widely separated dose levels are compared. The estimates of

TABLE 1

POTENCIES OF VAL^3-OXYTOCIN AND PHE^3-OXYTOCIN DETERMINED ON RAT UTERI *IN VITRO* AND AT DIFFERENT DOSE LEVELS

The stated dose of the synthetic analogues is the mean of the high and low dose used in the assay, oxytocin being used as standard

Assay method used	Val^3-oxytocin		Phe^3-oxytocin	
	Dose m-u.	Potency u./ml.	Dose m-u.	Potency u./ml.
Uterus in organ bath	48·0	0·9	24·0	3·7
	1·5	2·0	2·5	3·7
Superfused uterus	0·045	5·3	0·1	6·6

potencies of oxytocin analogues obtained from assays on superfused rat uteri disagreed also with those obtained from experiments on uteri suspended in an organ bath. The potencies estimated for the analogues were greater in the superfusion assays (Table 1).

All the preparations used contained pharmacologically inert material, and there was the possibility that some of these discrepancies in the potency estimates were due to the presence of different impurities. To investigate this possibility, val^3-oxytocin and phe^3-oxytocin were partially purified by ascending paper chromatography. In the case of val^3-oxytocin two spots were revealed ; the oxytocic activity was found in eluates from the faster-moving spot. Phe^3-oxytocin yielded only one spot and the oxytocic activity was found in this region only. To part of the eluate containing the oxytocic activity, eluate from the remainder of the paper strip was added, and this mixture was compared with the eluate from the " active spot " in assays on a rat uterus in an organ bath. The presence of impurities did not affect the oxytocic potency of either of the partially purified oxytocin analogues. Apart from the possibility of impurities which move at the same rate as the oxytocins on the paper, it is unlikely that the discrepancies in the relative potency estimates for the oxytocin analogues were due to any impurities in the solutions.

DISCUSSION

The fate of val^3-oxytocin and phe^3-oxytocin in intact male rats has been shown to be qualitatively similar to that of natural oxytocin, being determined by uptake by the kidneys and organs of the splanchnic vascular area. However, the rate of disappearance of val^3-oxytocin from the circulation was much slower than that of oxytocin or phe^3-oxytocin. This longer persistence of val^3-oxytocin in the circulation was also seen in nephrectomized male rats without splanchnic circulation in which

the distribution of the other two peptides quickly proceeded to equilibrium, suggesting that the slow decline in the blood concentration of val^3-oxytocin may be due to a permeability barrier. The potency per unit weight of val^3-oxytocin is not known, but if its potency was low the amounts injected might have been great enough to exceed the capacity of some part of an inactivating system. However, the weight of the phe^3-oxytocin injected in each clearance experiment was about 20 times that of oxytocin (Boissonnas & Huguenin, 1960), and yet the rate of disappearance was not slower than that of the naturally occurring hormone. If permeability is the critical factor, the barrier which can differentiate between val^3-oxytocin and phe^3-oxytocin or oxytocin must possess a high degree of specificity.

We have confirmed the findings of Boissonnas *et al.* (1956) and Berde, Doepfner & Konzett (1957) that the potency ratio, val^3-oxytocin : oxytocin, is greater when measured *in vivo* than when the assay is performed on a rat uterus in an organ bath. The longer persistence of val^3-oxytocin in the circulation and its longer duration of action cannot account for its greater potency, which is measured by the maximum increase in tonus, that is, an effect which occurs 1 to 2 min after injection. Furthermore, the difference between the potencies of phe^3-oxytocin *in vivo* and *in vitro* is much more marked than those of val^3-oxytocin, although the rate of disappearance and duration of action of the latter compound could not be differentiated from those of oxytocin. Since the potency of val^3-oxytocin *in vitro* is enhanced in the presence of 0.5 mM/l. magnesium, Munsick (1960) has suggested that the difference between the potency of oxytocin and val^3-oxytocin *in vitro* and *in vivo* may only arise when magnesium is absent from the bath solution. However, the potency of phe^3-oxytocin was 12 times greater when measured *in vivo,* but Munsick (1960) found that its *in vitro* activity was only slightly enhanced when the solution had high magnesium content.

The response of an individual uterus under the conditions of the British Pharmacopoeial assay to a given dose of val^3-oxytocin or phe^3-oxytocin can be matched by a dose of oxytocin, but, to match the response of a rat uterus *in situ* to a dose of one of the oxytocin analogues, more oxytocin is needed than could be expected from the *in vitro* results. That is, the responsiveness of the rat uterus *in situ* to the various oxytocins is different from that *in vitro* and the degree of change in response varies from substance to substance. On a weight basis, phe^3-oxytocin is about 20 times less potent than oxytocin *in vitro,* but *in vivo,* in rats, oxytocin is only 1.8 times more potent than the synthetic analogue.

The unit of oxytocic activity is defined in terms of a definite weight of the international standard powder (of which the oxytocic activity is primarily but not solely due to oxytocin), and any assay preparation in which oxytocin is compared with an analogue should give a potency which could be expressed in international units of oxytocic activity. Munsick (1960) has shown that the results of *in vitro* assays comparing oxytocin with an analogue may depend upon the composition of the suspension fluid, particularly with respect to its magnesium content. We have found that the result may also vary with the magnitude of the response used in the assay itself. It would therefore appear that, owing to the fundamental invalidity of all these assays, in that the standard is not identical with the unknown, there is

no definite amount of an oxytocin analogue which could be equivalent to a unit of oxytocic activity. This difficulty is not realized by most workers, who arbitrarily make the assay on the rat uterus *in vitro* the reference assay upon which the unit of oxytocic activity now, tacitly, depends. It would be better, perhaps, if this situation were recognized and the unit redefined.

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POTENTIATION OF SUBSTANCE P BY LYSERGIC ACID DIETHYLAMIDE IN VIVO

BY

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In doses of 10 μg/kg or more, lysergic acid diethylamide enhanced the fourth potential (DR IV) of the dorsal root potential complex in the cat. Smaller doses of lysergic acid diethylamide did not in themselves alter the DR IV, but revealed an enhancement of the potential by substance P, which by itself had no effect. 2-Bromolysergic acid diethylamide had no action on the dorsal root potentials, but prevented the actions of lysergic acid diethylamide.

The fact that posterior spinal roots contain large amounts of substance P and contain little or no acetylcholine led Lembeck (1953) to suggest that substance P is the chemical transmitter liberated by the first sensory neurone. If this were true, the administration of substance P might be expected to modify spinal reflexes. However, substance P is destroyed by an enzyme system which is found in various parts of the body (Gulbring, 1943), including spinal cord (Lembeck, 1953). The presence of this enzyme precludes examination of Lembeck's suggestion by simply determining the effect of injected substance P whilst examining reflex activity, unless extremely large amounts of substance P are used. Krivoy (1957) confirmed Gulbring's observations and also found that lysergic acid diethylamide was a specific antagonist to the enzyme responsible for destroying substance P. The purpose of the present experiments was to determine if, by using lysergic acid diethylamide as an enzyme inhibitor *in vivo*, it might be possible to unmask the actions of small amounts of substance P on transmission of impulses from the posterior spinal root to secondary and internuncial neurones of the spinal cord.

METHODS

The technique of Lloyd & McIntyre (1949) was used to evaluate the actions of substance P on transmission of nerve impulses along the intramedullary pathway of the primary afferent fibre to secondary neurones. These authors recorded and analysed five dorsal root potentials (DR I, II, III, IV, V) from a dorsal spinal rootlet adjacent to another stimulated rootlet (Fig. 1). DR I II, and III are due to intramedullary conduction of the nerve impulse along the primary afferent nerve. DR IV is mainly due to a residual negativity associated with supernormality in the nerve endings of the primary afferent fibre, as well as in the secondary neurone (see also Rudin & Eisenman, 1953).

Twenty-three decerebrate cats were used in this study: in addition to decerebration, four of these cats had spinal transections at L1. All operative procedures were performed under

ether anaesthesia. The last lumbar or first sacral spinal rootlets were used in all experiments. The entire area of exposed spinal cord and rootlets was covered with liquid paraffin contained in a trough constructed of the incised skin. The liquid paraffin was previously equilibrated with carbon dioxide and maintained at 37° C by radiant heat.

Stimuli used were biphasic (square waves of 50 μsec duration coupled through a 2 μF condenser) at a frequency of 0.5 or 2.5 cycles/sec. Intensity was adjusted so as to be either maximal or about 50% of maximal for DR IV. Stimulation, once started, was maintained without interruption for the duration of the experiment. Conventional electrophysiological techniques were used for amplification and display of the evoked potentials.

The drugs used in this study, lysergic acid diethylamide, 2-bromolysergic acid diethylamide and substance P, were injected via a polythene cannula in the femoral vein. No drug was given until at least 1 hr after the termination of ether anaesthesia, and then only after the size of the dorsal root potentials had remained constant for at least 30 min.

RESULTS

Stimulation at 2.5 cycles/sec. With submaximal stimuli, lysergic acid diethylamide, 5 μg/kg, enhanced DR IV in approximately half of the cats studied; 10 μg/kg produced enhancement in every cat. The augmentation of DR IV appeared within 1 min of the time of injection and reached a maximum approximately 5 min thereafter; if at this time the stimulus intensity was reduced so that DR IV was again submaximal, DR IV remained at this level, indicating that the action of lysergic acid diethylamide had in fact reached a plateau, and there had been no background of continued enhancement. The administration of large amounts of lysergic acid diethylamide did not alter the qualitative nature of the response, and, even with the administration of 120 μg/kg, only enhancement was seen.

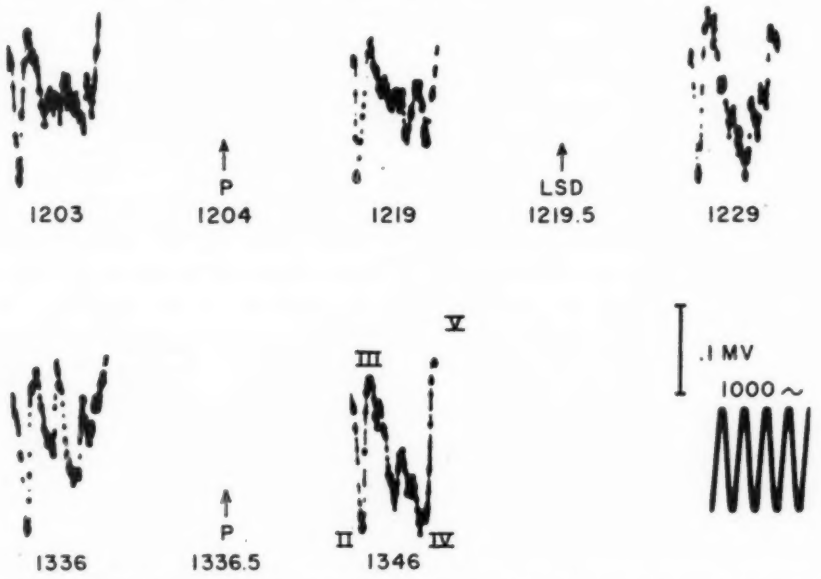

Fig. 1. Decerebrate cat. Dorsal root potentials evoked by biphasic stimuli (square waves of 50 μsec duration coupled through a 2 μF condenser). Injections of substance P (P) (30 u./kg at 1204 and 12 u./kg at 1336.5) and lysergic acid diethylamide (5 μg/kg) (LSD) were given via the femoral vein. The time is indicated under each tracing. The Roman numerals in the final trace indicate the designation of the potential sequence.

Substance P alone had no action on the dorsal root potentials when as much as 30 u./kg was given. On the other hand, when substance P, 12 u./kg, was given after 5 μg/kg of lysergic acid diethylamide, it produced enhancement of DR IV. This response to substance P was observed if the previous administration of lysergic acid diethylamide had not produced its own action, or if, after obtaining enhancement, the stimulus intensity was reduced so as to render DR IV approximately equal to its control value relative to maximal. The enhancement of DR IV under these conditions could not be distinguished from the enhancement of DR IV produced by effective doses of lysergic acid diethylamide (Fig. 1).

Occasionally DR I, II, III and V were found to increase after DR IV had increased in response to lysergic acid diethylamide or to lysergic acid diethylamide followed by substance P. Bromolysergic acid diethylamide in doses up to 100 μg/kg had no action on dorsal root potentials ; lysergic acid diethylamide after bromolysergic acid diethylamide was found to have no action on dorsal root potentials in doses up to 100 μg/kg. There was no observable difference in response between decerebrate cats and decerebrate-spinal cats. With maximal stimuli, lysergic acid diethylamide in extremely large doses (70 μg/kg) produced enhancement of DR IV.

Submaximal or maximal stimuli at 0.5 cycles/sec. Lysergic acid diethylamide, substance P and combinations of these two drugs were found to have no clear-cut action on the preparation.

DISCUSSION

As the present results were obtained both in the decerebrate cat and in the decerebrate-spinal cat, the phenomena described must be mediated by an action directly on the spinal cord, rather than being secondary to events occurring more centrally. Lysergic acid diethylamide had no action at low frequencies of stimulation. This could be explained if lysergic acid diethylamide acts on the spinal cord by preventing the enzymatic degradation of a neurohormone, for its action should appear most clearly when that neurohormone is being produced in large amounts and rapid destruction is most critical, that is, during a period of rapid stimulation when the opportunity for temporal summation is greatest. In view of the enzymatic destruction of substance P, it is not surprising that the amounts of substance P used here had no action of their own (Kissel & Domino, 1959). The fact that large amounts of lysergic acid diethylamide produce the same phenomena as smaller doses of lysergic acid diethylamide and substance P would indicate a mechanism analogous to the phenomena one sees with the anticholinesterases and acetylcholine. One wonders if the potentiation bears any relation to the potentiation of ganglionic transmission by substance P reported by Beleslin, Radmanović & Varagić (1960).

The observation that substance P depresses spinal reflexes (Stern & Dobrič, 1957) may represent the second phase of a biphasic action of substance P on spinal transmission. Thus, substance P, like acetylcholine, may stimulate in small concentrations and depress in larger ones. Morphine antagonism by substance P (Zettler, 1956) can also be explained. Whereas substance P augments DR IV, morphine inhibits DR V (Krivoy & Huggins, unpublished), so that the polypeptide enhances the flow of sensory impulses which are antagonized at a later adjacent site by morphine.

The observation that 2-bromolysergic acid diethylamide does not change the dorsal root potentials but inhibits the action of lysergic acid diethylamide is additional evidence that bromolysergic acid diethylamide does not protect substance P from enzymatic destruction, but antagonizes the actions of lysergic acid diethylamide (Krivoy, 1957). Ginzel & Mayer-Gross (1956) also found that, in man, pretreatment with 2-bromolysergic acid diethylamide renders lysergic acid diethylamide ineffective.

Potentiation of DR I, II, III, in the cases where it was observed, was probably secondary to potentiation of DR IV (Grunfest & Magnes, 1951).

The data presented in this paper not only support the concept that substance P is a neurohormone, but indicate that it possesses a role in sensory transmission.

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EFFECTS OF 5-HYDROXYTRYPTAMINE ON THE DORSAL MUSCLE OF THE LEECH (HIRUDO MEDICINALIS)

BY

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5-Hydroxytryptamine has an inhibiting effect on the leech muscle. It reduces the contractions produced by acetylcholine or nicotine and accelerates the relaxation of the muscle when these substances are washed out. This acceleration of relaxation allows a more rapid assay of acetylcholine in this preparation.

It has been found by Poloni that 5-hydroxytryptamine causes relaxation of the isolated leech muscle and that this effect can be utilized for its assay in concentrations as low as 10^{-12} g/ml. (Poloni, 1955b). Poloni had reported earlier that samples of cerebrospinal fluid from schizophrenics relax the leech muscle (Poloni, 1951). He suggested that 5-hydroxytryptamine was the active principle in these fluids (Poloni, 1955a).

The present experiments confirm the relaxing effect of 5-hydroxytryptamine on the leech muscle and describe some further actions of this substance on this preparation. However, the relaxing effect has not been found suitable as a basis for the assay or identification of 5-hydroxytryptamine.

METHODS

The experiments were carried out on twin strip preparations of the dorsal muscle of the leech *Hirudo medicinalis*. The muscle strips were suspended in 5 ml. mammalian Locke solution diluted 1 to 1.4 with distilled water. Eserine sulphate was added to the bath fluid at concentrations of 5 to 10 μg/ml. The bath temperature was kept at about 20° C. The method is described in detail by MacIntosh & Perry (1950).

The compounds tested on the leech muscle were acetylcholine chloride, 5-hydroxytryptamine creatinine sulphate, nicotine hydrogen tartrate, potassium chloride and (+)-lysergic acid diethylamide. All values refer to the salts. The acetylcholine, nicotine and potassium were left in contact with the muscle for 60 to 90 sec. When the actions of 5-hydroxytryptamine or (+)-lysergic acid diethylamide were being studied, their concentrations were often maintained by adding them to the bath after each washing.

An attempt was made to determine if 5-hydroxytryptamine could be normally found in leech tissue. Extracts were made by mincing whole leeches and extracting them with 80% acetone, 25 ml. per leech, for 24 hr at 4° C. After filtration, the acetone was evaporated off under a vacuum pump and the residue re-suspended in 3 ml. distilled water.

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RESULTS

Fig. 1 shows the contractions produced by 3 ng/ml. of acetylcholine and their reduction on the addition of 5-hydroxytryptamine (200 ng/ml.) to the bath. After washing out the 5-hydroxytryptamine together with the acetylcholine, the sensitivity of the muscle to acetylcholine gradually returns. Besides diminishing the size of the acetylcholine contraction, the inhibitory effect of 5-hydroxytryptamine is evident

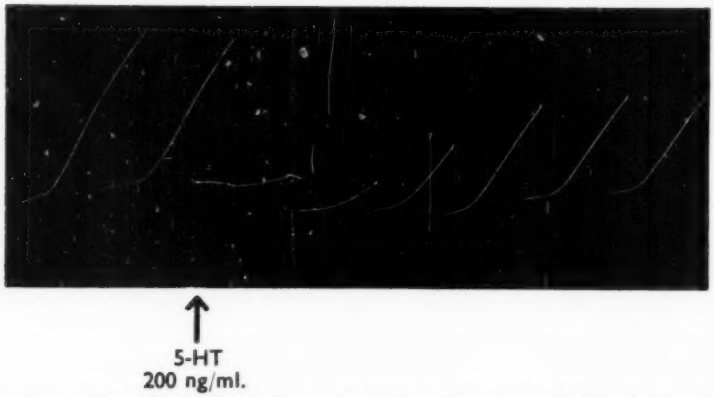

Fig. 1. Contraction of the eserinized leech muscle to 3 ng/ml. acetylcholine given at 5 min intervals and left in the bath for 90 sec. Three minutes before the third addition of acetylcholine (at arrow), 200 ng/ml. of 5-hydroxytryptamine was added to the bath and washed out 4.5 min later together with the acetylcholine.

by the fact that it accelerates relaxation of the muscle after washing out the acetylcholine. The relaxing effect is also seen in a muscle not contracted by acetylcholine, but the effect is less pronounced. This small relaxation has not been found suitable for the assay of 5-hydroxytryptamine because of the difficulty in establishing a stable base line for comparison of relaxations.

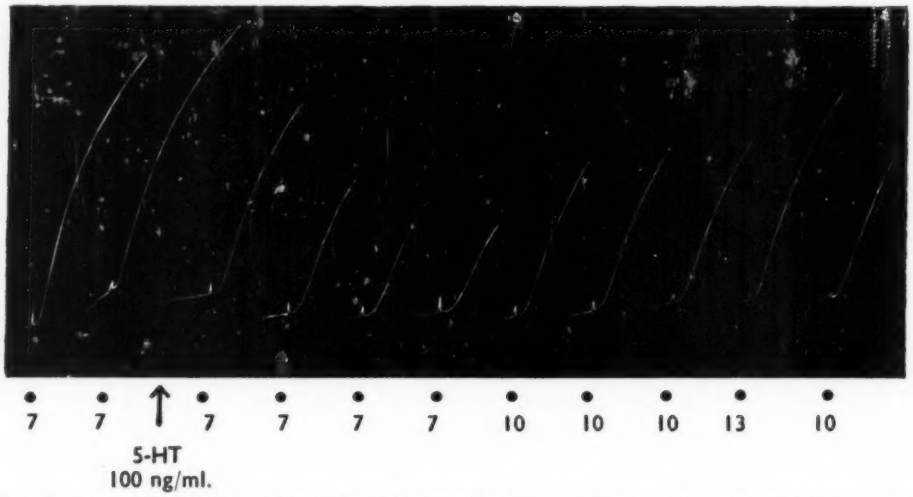

Fig. 2. Contraction of the eserinized leech muscle to varying amounts of acetylcholine given at 5 min intervals. At the arrow, 100 ng/ml. 5-hydroxytryptamine is added to the bath and again after each renewal of the bath fluid. The figures below the contractions refer to the amounts of acetylcholine in ng/ml. added to the bath.

The threshold concentration of 5-hydroxytryptamine sufficient to reduce the size of contractions produced by 3 to 5 ng/ml. of acetylcholine is between 25 and 50 ng/ml. When the 5-hydroxytryptamine is kept in the bath fluid, the degree of inhibition increases during the first 20 to 30 min and then becomes stable as shown in Fig. 2. The time required for the recovery from the effect of 5-hydroxytryptamine is dependent on the time the 5-hydroxytryptamine has been in contact with the muscle. At least 1 hr is required for full recovery of the acetylcholine sensitivity of the tissue following contact with 5-hydroxytryptamine for half an hour.

The relaxing effect of 5-hydroxytryptamine can be utilized to perform the acetylcholine assay more rapidly on the leech muscle. The presence of 100 to 300 ng/ml. of 5-hydroxytryptamine in the bath fluid renders it possible to elicit acetylcholine contractions at about 5 min intervals without undue changes in the base line. In

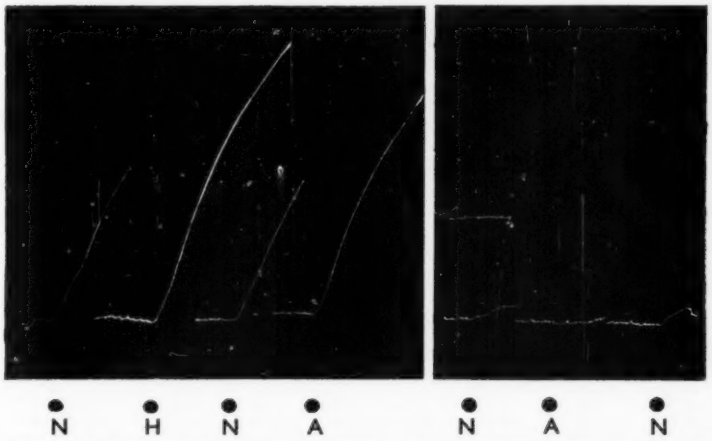

Fig. 3. Contractions of the eserinized leech muscle to 400 ng/ml. nicotine (N) and to 2[ng/ml. acetylcholine (A). Between the two tracings, there is an interval of 30 min during which 400 ng/ml. of 5-hydroxytryptamine was kept in the bath.

the experiment of Fig. 2, the acetylcholine is given for 90 sec at 5 min intervals. It can be seen that in the presence of 100 ng/ml. 5-hydroxytryptamine, the base line remains relatively constant and that there is good discrimination between 7, 10 and 13 ng/ml. of acetylcholine.

In contrast to 5-hydroxytryptamine, lysergic acid diethylamide does not reduce acetylcholine contractions even if added to the bath in amounts up to 1 μg/ml. With this concentration of lysergic acid diethylamide, a slight relaxation of the muscle is usually obtained.

The nicotine contractions of the leech muscle are inhibited by 5-hydroxytryptamine in the same way as are acetylcholine contractions (Fig. 3), but potassium contractions are affected to a much lesser degree (Fig. 4).

Acetone extracts of the leech contracted the rat uterus and the guinea-pig ileum preparations. The effects were obtained by the addition to the 5 ml. bath of 0.05 ml. extract corresponding to about 35 mg of fresh tissue. The contractions were not due to acetylcholine, histamine or 5-hydroxytryptamine, since they were not abolished by atropine, mepyramine or lysergic acid diethylamide. They were not

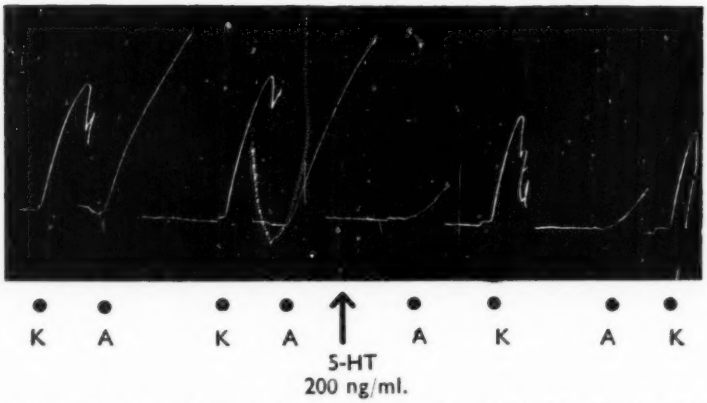

Fig. 4. Contractions of the eserinized leech muscle to 0.3 mg/ml. of potassium chloride (K) and to 2 ng/ml. of acetylcholine (A). From the arrow until the end of the record, the bath contained 200 ng/ml. of 5-hydroxytryptamine.

due to potassium, since the extracts became inactive upon ashing; nor due to bradykinin, since they remained active after incubation with chymotrypsin. No further attempt was made to identify the active principle, or principles, in the extracts.

DISCUSSION

Relaxing effects of 5-hydroxytryptamine on smooth muscle preparations have been reported by Brecht & Jeschke (1960) on the isolated frog lung and by Twarog (1954, 1960) on the molluscan retractor muscle. According to Twarog, 5-hydroxytryptamine prevents the maintenance of tension which develops in response to acetylcholine, but the initial tension developed is potentiated. This author therefore suggests that 5-hydroxytryptamine may be involved in a tension-maintaining system of molluscan smooth muscle that is independent of the tension-developing system. In the leech muscle, both development and maintenance of tension were found to be inhibited by 5-hydroxytryptamine.

In an earlier report, Erspamer (1955) failed to find appreciable quantities of 5-hydroxytryptamine in the ventral ganglionic chain of the leech using paper chromatography and bioassay techniques. Our finding, using less refined dissection methods, was the same. However, recently Welsh & Muirhead (1960) made an extensive survey of the distribution of 5-hydroxytryptamine in invertebrates. They measured the 5-hydroxytryptamine content of pooled nerve cords dissected out from leeches, and found 6.9 μg of 5-hydroxytryptamine per gramme of fresh tissue. This was a relatively large amount of 5-hydroxytryptamine as compared to the content in neural tissues of other invertebrate species examined.

On the basis of the sensitivity of muscles of crustaceans and molluscans to 5-hydroxytryptamine and the presence of this substance in neural tissue of these classes, Welsh (1957) has proposed that 5-hydroxytryptamine has a neurohumoral role in the physiology of invertebrates. The relaxing effect of 5-hydroxytryptamine upon the annelid *Hirudo medicinalis* is another example of the responsiveness of invertebrate organs to this substance.

At present, it seems to be not justifiable to attribute the relaxation of leech muscle produced by samples of cerebrospinal fluid from schizophrenic patients to an action of 5-hydroxytryptamine. Other substances also cause relaxation of the leech muscle such as guanidine (Fühner, 1918), adenylic acid (Minz, 1932), and potassium chloride in threshold concentrations (Vartiainen & Kostia, 1937). Furthermore, the presence of 5-hydroxytryptamine in the cerebrospinal fluid of schizophrenics has not been confirmed by other workers using different methods of assay (Schain, 1960).

I wish to thank Sir Charles Harington for hospitality, and Professor W. Feldberg for his interest.

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ACTIONS OF AMINO-ACIDS ON THE ISOLATED HEMISECTED SPINAL CORD OF THE TOAD

BY

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The actions of a series of amino-acids related to γ-aminobutyric acid and glutamic acid have been determined upon the isolated and sagittally hemisected spinal cord of the toad, *Bufo marinus*. Slow and fast components of the ventral root reflex responses following dorsal root stimulation were depressed by γ-aminobutyric acid and by a series of neutral amino-acids having a similar structure. The relative depressant potencies of the members of this series were determined by comparison of the concentrations of each required to cause the same reduction in the electrically integrated slow components of these reflex responses. Glutamic acid and closely related substances facilitated reflex responses in low concentrations and depressed these responses in high concentrations. The actions of these substances resulted in the depolarization of motoneurones which was recorded as a negative potential in the ventral root. The relative potencies of the substances were estimated from the concentrations of each required to produce negative potentials of the same magnitude.

Several amino-acids not previously tested proved to have remarkably strong actions on this preparation. 3-Aminopropanesulphonic acid was the most potent depressant tested; homocysteic acid and N-methylaspartic acid were the most powerful excitatory substances. The D forms of optically active depressants or excitants were always stronger than the corresponding L forms where both enantiomorphs were available. Points of similarity and dissimilarity between these results and those of related investigations are discussed.

Much interest has centred around the recently revealed ability of certain amino-acids to modify electrical events recorded at neuronal and neuromuscular junctions. These amino-acids can be classified into two broad groups: those which decrease and those which increase the excitability of post-synaptic elements (Curtis & Watkins, 1960). Substances of the latter group, in concentrations higher than those required to produce excitation, may also cause depression (Brockman & Burson, 1957; Robbins, 1959; Van Harreveld, 1959; Curtis, Phillis & Watkins, 1960), but such depression is clearly distinguishable from that manifested at all concentrations by substances of the first group (Curtis, Phillis & Watkins, 1959; Curtis *et al.*, 1960). Amongst the most potent members of these two groups of amino-acids, which can for convenience be termed the depressant and excitant series, are γ-aminobutyric acid and glutamic acid respectively. These two compounds bear a simple structural relationship to one another, which, for substances applied iontophoretically into the environment of single cells in the cat's spinal cord, is characteristic of members of the two series (Curtis & Watkins, 1960). Certain differences in the general picture of structure–activity relationships have, however, emerged from studies involving

various other tissues and test situations (Purpura, Girado, Smith, Callan & Grundfest, 1959; Edwards & Kuffler, 1959; Van Harreveld, 1959). In particular, several substances which were inactive when applied iontophoretically to single cells in the cat's spinal cord have been reported to have strong actions, either depressant or excitant, on the responses of groups of cells elsewhere in the nervous system when topically applied. It was with the dual purpose of further investigating such differences, and of developing methods for comparing quantitatively the potencies of groups of closely related depressant and excitant substances, that the present work was undertaken. The opportunity was also taken to test, for the first time, several previously unavailable synthetic amino-acids, some of which proved to be remarkably potent on this particular preparation.

METHODS

All experiments were performed on segments of the isolated spinal cord of the Queensland toad, *Bufo marinus*. The cord was hemisected sagittally and was fixed in a small trough of 1.5 ml. capacity which could be perfused with amphibian Ringer solution of the following composition: NaCl, 100 mM; KCl, 2.5 mM; Na_2HPO_4, 2.5 mM; NaH_2PO_4, 0.45 mM; $CaCl_2$, 1.9 mM; $NaHCO_3$, 12 mM; glucose, 2.8 mM. This solution was equilibrated with a mixture of 95% oxygen and 5% carbon dioxide before delivery into the trough and its *p*H was 7.4. Both cervical and lumbar regions of the spinal cord were used and appropriate dorsal and ventral roots were drawn through small holes in the lateral walls of the trough into pools of liquid paraffin B.P. The trough and paraffin compartments formed portion of the top of a water jacket through which water at $15 \pm 0.5°$ C was circulated. To ensure that the Ringer and test solutions were delivered to the trough at the same temperature, these solutions were passed through coils of polythene tubing immersed in the water jacket. The rate of flow of the Ringer solution between applications of test substances was about 3 ml./min. No apparent changes took place in the preparation when the circulation of the Ringer solution was interrupted for periods as long as 15 min.

The response of the tissue to an amino-acid was determined from potentials recorded from the ventral roots. Spike potentials and electrotonically propagating slow potentials were recorded monophasically between two non-polarizable electrodes (Ag-AgCl), one being in the trough of Ringer solution and the other at the crushed end of the ventral root. The effective lengths of the ventral roots were usually about 2 to 4 mm in cervical preparations and 12 to 14 mm for a lumbar preparation. Electrical stimuli were applied to dorsal roots by means of a pair of platinum electrodes immersed in the paraffin pool. Two amplifiers were available for recording the subsequent ventral root responses on the oscilloscope, these having time constants of 5 msec and 3 sec. The former was suitable for recording the spike components of the reflex, but the latter was necessary to demonstrate the slower components. In many of the experiments the reflex response was also electrically integrated over a period of 600 msec following the stimulus, the integrator having a time constant of 10 sec. A direct-coupled amplifier was used, in conjunction with either an oscilloscope or a pen-recorder, to record the potentials produced in the ventral root by an amino-acid in the absence of dorsal root stimulation.

The substances to be tested were dissolved in Ringer solution, equilibrated with 95% oxygen and 5% carbon dioxide and the *p*H readjusted to 7.4, where necessary, with N sodium hydroxide or N hydrochloric acid. The flow of Ringer solution through the trough was interrupted immediately before the application, and the fluid retained by the trough was rapidly flushed out with three to four volumes of the test solution.

The relative potencies of the actions on the isolated toad cord of a series of depressant compounds related to γ-aminobutyric acid were determined by alternately applying a standard solution of 10^{-3} M γ-aminobutyric acid and a solution of a particular test substance. The

concentration of the test substance was varied until the diminution of the integrated ventral root response produced by the γ-aminobutyric acid and test solutions was the same. Applications of standard and test solutions were each of 1 min duration, the interval between successive applications being 15 min. Experiments were carried out on at least one lumbar and three cervical preparations for each substance. The relative potencies of the substances were approximately the same on each tissue, irrespective of the particular region which was used.

For comparison of the potencies of excitant compounds, 5×10^{-4} M L-glutamic acid was used as the standard. This solution and solutions of each particular test substance were applied alternately, the concentration of the test substance being varied until the magnitude of the negative potential developed in the ventral root was the same as that produced by the standard glutamic acid solution. Applications were each of 45 sec duration, and the interval between successive applications was again 15 min. To facilitate the comparisons, the potentials developed in the ventral root were monitored by a paper recorder after suitable amplification with a direct-coupled amplifier. As with the depressants, most of the excitant substances were tested both on cervical and lumbar segments, there again being no significant differences in the relative potencies of the substances observed in the two series. No substance, either excitant or depressant, was tested in a concentration greater than 5×10^{-2} M; if a substance was without effect at this concentration, it was considered to be inactive.

Interpretation of recorded potentials

A volley entering the toad spinal cord in one of the dorsal roots produces a complex response in the corresponding and adjacent ventral roots (Eccles, 1946, 1947; Brookhart, Machne & Fadiga, 1959). The post-synaptic potentials produced in motoneuronal somas by mono- and poly-synaptic pathways (Araki, Otani & Furukawa, 1953; Machne, Fadiga & Brookhart, 1959; Araki, 1960) propagate electrotonically along the axons and are detected by a recording electrode situated on a ventral root as a negative potential. This potential may take several hundred milliseconds to reach a maximum and subside, the actual time depending on the strength and frequency of stimulation. Superimposed upon these slow potentials are fast spike potentials conducted along the axons of those motoneurones in which the excitatory post-synaptic potentials attained threshold level. Such composite responses of the ventral root were evoked by stimulating the dorsal root at frequencies of 12/min or less, the strength of the stimulus being selected to produce a slow ventral root potential reaching a maximum value of about 1 to 2 mV. A reliable estimate of the magnitude of these slow potentials can be obtained by integration. With the low frequencies of stimulation used the integrated response remained practically constant for several hours.

These slow negative potentials recorded from the ventral root are a measure of the depolarization of all motoneurones whose axons lie in that particular root. The depolarization which follows dorsal root stimulation represents the sum of excitatory post-synaptic potentials produced monosynaptically by impulses in afferent fibres, together with excitatory and inhibitory post-synaptic potentials produced by interneurones which are activated by the dorsal root volley and which synapse with the motoneurones. When chemical substances are applied to this complex tissue in the absence of dorsal root stimulation, they may affect motoneurones, interneurones, or both of these groups of cells simultaneously. The net effect of such substances upon motoneurones, as determined by the potentials recorded from the ventral root, will depend upon which cells are affected, actions upon interneurones only being important if the cells are actually caused to discharge and thus to generate, either directly or after relay, excitatory or inhibitory post-synaptic potentials upon the membrane of the motoneurones with which they synapse. Thus, negative or positive potentials will be recorded from the ventral root depending upon whether motoneurones are directly depolarized or hyperpolarized by a particular substance, and upon whether the substance causes the discharge of excitatory or inhibitory interneurones. These potentials thus provide evidence of neuropharmacological activity, but give no information as to the original site or mechanism of this action. For such information, it would be necessary to carry out more definite experiments, such as those involving iontophoretic application of the substances to single cells.

RESULTS

γ-Aminobutyric acid

Action on the ventral root responses following dorsal root stimulation. Fig. 1 illustrates the depressant action of γ-aminobutyric acid on the reflex responses of the isolated hemisected spinal cord of the toad. The comparatively short latency components of the reflex responses were recorded from the ventral root of the second and third segments after stimulation of the corresponding dorsal root. After the control response of Fig. 1 A, a solution containing 5×10^{-4} M γ-aminobutyric acid was applied, and the responses B and C were recorded 30 and 80 sec later respectively. The bath was then flushed with Ringer solution, and the preparation had fully recovered 150 sec later (D). Higher concentrations of γ-aminobutyric acid caused greater depression; Fig. 1, E, F, G, H, and I, J, K, L, illustrates respectively the effects of 10^{-3} M and 5×10^{-3} M on the same reflex response. E and I are control responses. Responses F and G were recorded 30 and 75 sec after the application of 10^{-3} M γ-aminobutyric acid, the preparation requiring 2 min for recovery (H). Responses J and K were recorded 10 and 30 sec after application of 5×10^{-3} M γ-aminobutyric acid, full recovery (L) in this case taking 6 min.

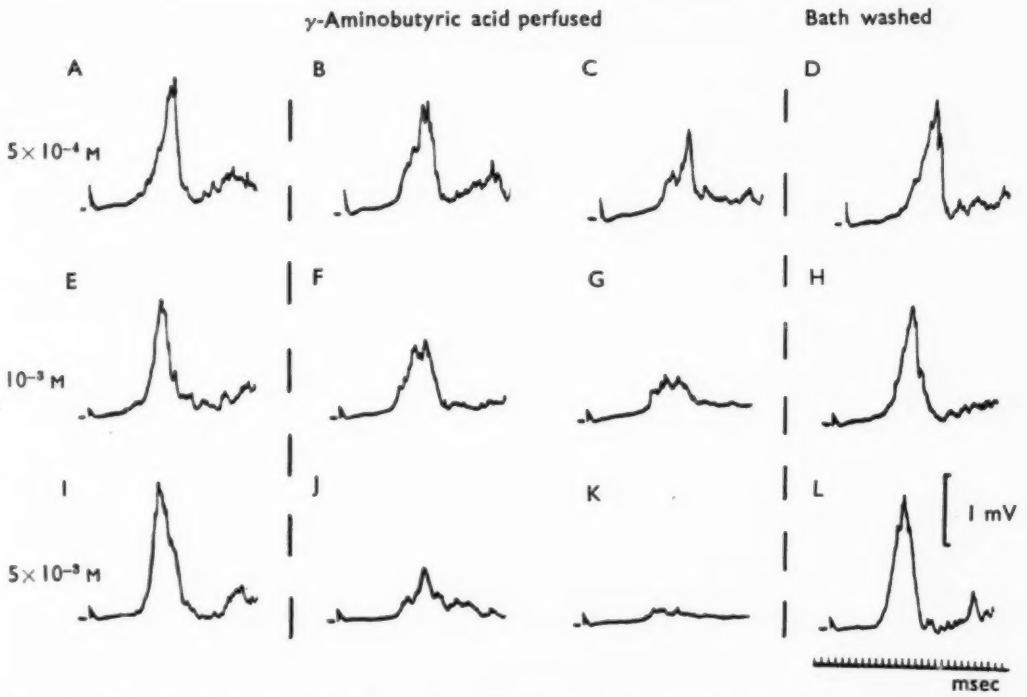


Fig. 1. Reflex discharges recorded from the ventral root of the second and third cervical segments in response to stimulation of the corresponding dorsal root. The intensity of the stimulus remained constant throughout the series. A, E, and I, control responses; B and C were recorded 30 and 80 sec after the commencement of perfusion with 5×10^{-4} M γ-aminobutyric acid; F and G, after 30 and 75 sec of perfusion with 10^{-3} M γ-aminobutyric acid; J and K, after 10 and 30 sec of perfusion with 5×10^{-3} M γ-aminobutyric acid; D, H, and L were recorded 150, 120, and 360 sec respectively in the corresponding series after washing the bath with amphibian Ringer solution. There was an interval of 15 min between each series. Time marker, msec. Voltage calibration, 1 mV.

The effects of γ-aminobutyric acid on both the faster and slower components of the ventral root reflex following dorsal root stimulation are shown in Fig. 2. The upper responses were recorded using an amplifier of 3 sec time constant and show the slow potential superimposed upon which are the faster components. Records A i, B i and C i are the responses before, during and after the application of 10^{-3} M γ-aminobutyric acid, and demonstrate the marked depression of both fast and slow components of the reflex. The lower responses A ii, B ii and C ii are the electrically integrated records of the responses A i, B i and C i, and show the depression more clearly.

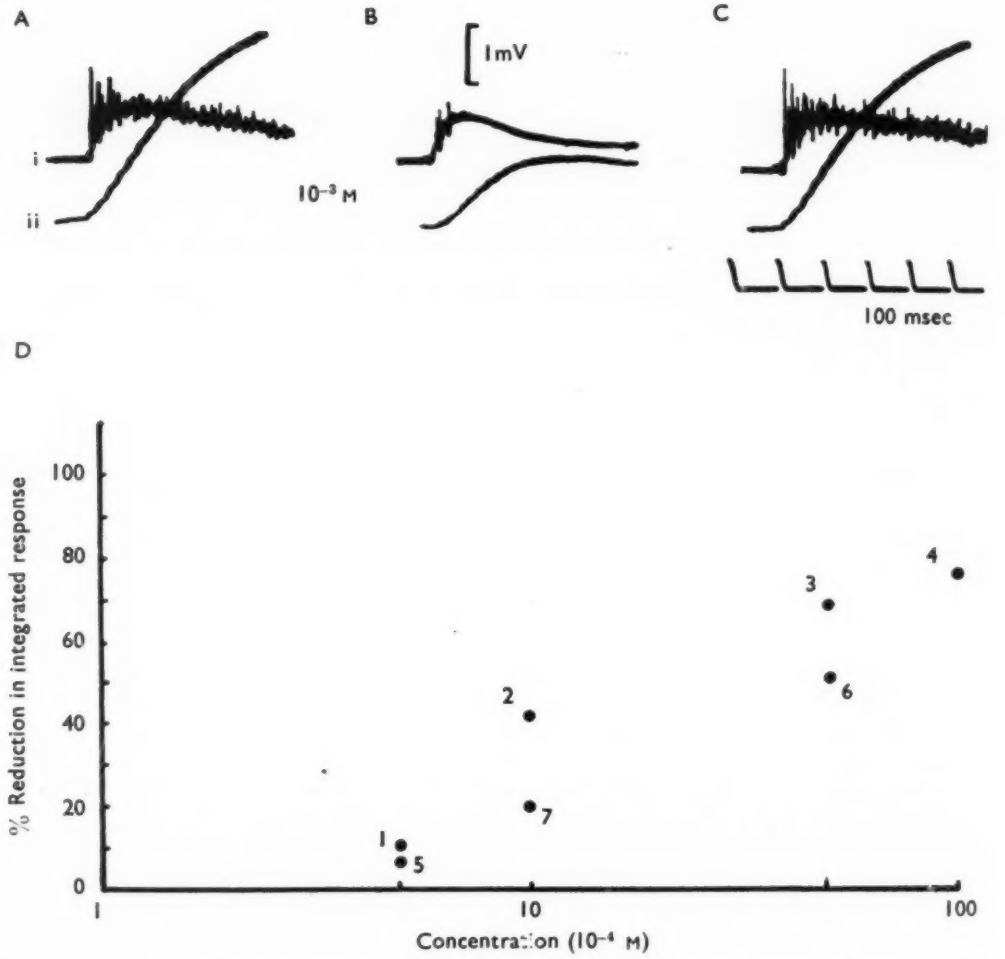


Fig. 2. A to C, Reflex responses evoked by dorsal root stimulation and recorded from the ninth spinal ventral root. i—Using an amplifier of 3 sec time constant. ii—Simultaneous record of the integral, with respect to time, of i. A—Control responses; B—after 55 sec perfusion with 10^{-3} M γ-aminobutyric acid; C—70 sec after washing the bath. Time—100 msec. Voltage calibration—1 mV for i. D, The depression of the integrated ventral root response produced by a series of concentrations of γ-aminobutyric acid, same preparation as A to C. Ordinate, percentage decrease of the integrated response relative to the control. Abscissa, concentration of γ-aminobutyric acid in the perfusing solution. All applications were of 50 sec duration and are numbered in the sequence of testing.

The depression produced by γ-aminobutyric acid was related to the concentration. It was found, however, that repeated applications of this drug in high concentration frequently resulted in a decline in depressant efficacy. These results are illustrated in Fig. 2 D. The points 1 to 4 represent the percentage reduction of the integrated ventral root response evoked by a dorsal root stimulus with a series of increasing concentrations of γ-aminobutyric acid. After the applications of this series, the solutions having the concentrations corresponding to points 1, 2 and 3 were re-tested, and each was found to give a much diminished depression of the integrated response (points 5, 7 and 6 respectively). In this experiment the applications were made in the sequence represented by the numerals; the time interval between successive applications was 15 min.

Potentials recorded from the ventral root in the absence of dorsal root stimulation. In most preparations, γ-aminobutyric acid, even in concentrations as high as 10^{-2} M, caused no significant potential change in the ventral root in the absence of dorsal root stimulation. In a few experiments, however, the drug elicited a positive potential of varying magnitude (mostly in the range of 0.5 to 1 mV with 10^{-3} M γ-aminobutyric acid), which indicated that the motoneuronal membranes had in these instances been hyperpolarized from the prevailing resting state. This effect seemed to be seasonal and may reflect changes in extra- and intra-cellular ionic concentrations (Machne *et al.*, 1959). It was also found that repeated applications of γ-aminobutyric acid to such preparations invariably resulted in a progressive diminution in the positive potential developed, and, often, ultimately to complete disappearance of the effect. Although this variable phenomenon did not influence significantly the observed relative potencies of a series of depressant compounds closely related to γ-aminobutyric acid, nevertheless, in order to maintain a greater uniformity of test conditions, only those spinal cords in which such a hyperpolarizing response was minimal were selected for comparative experiments.

L-*Glutamic acid*

Action on the fast components of the ventral root responses following dorsal root stimulation. Fig. 3 illustrates the effects of three progressively increasing concentrations of L-glutamic acid upon responses recorded from the ventral root of the isolated toad cord. The responses shown are those recorded from the ventral root following dorsal root stimulation (A to D, E to H and M to P) and in the absence of dorsal root stimulation (I to L). After the control response A, a solution containing 5×10^{-4} M glutamic acid was applied and the responses B and C recorded 10 and 50 sec later. The glutamic acid solution was then flushed out, the recovered response D being recorded 2 min later. The responses of Fig. 3 E to H are analogous to those of A to D, except that the concentration of L-glutamic acid was 5×10^{-3} M. These records show the marked increase in the fast components of the reflex and the depression of the slow component upon which the spikes were superimposed. Fig. 3, I to L, shows spike responses evoked in the ventral root by 5×10^{-3} M L-glutamic acid in the absence of dorsal root stimulation; the responses J to L were recorded at the same time intervals after the control (I) as those of A to D and E to H. The responses of Fig. 3, M to P, demonstrate the typical effect of high

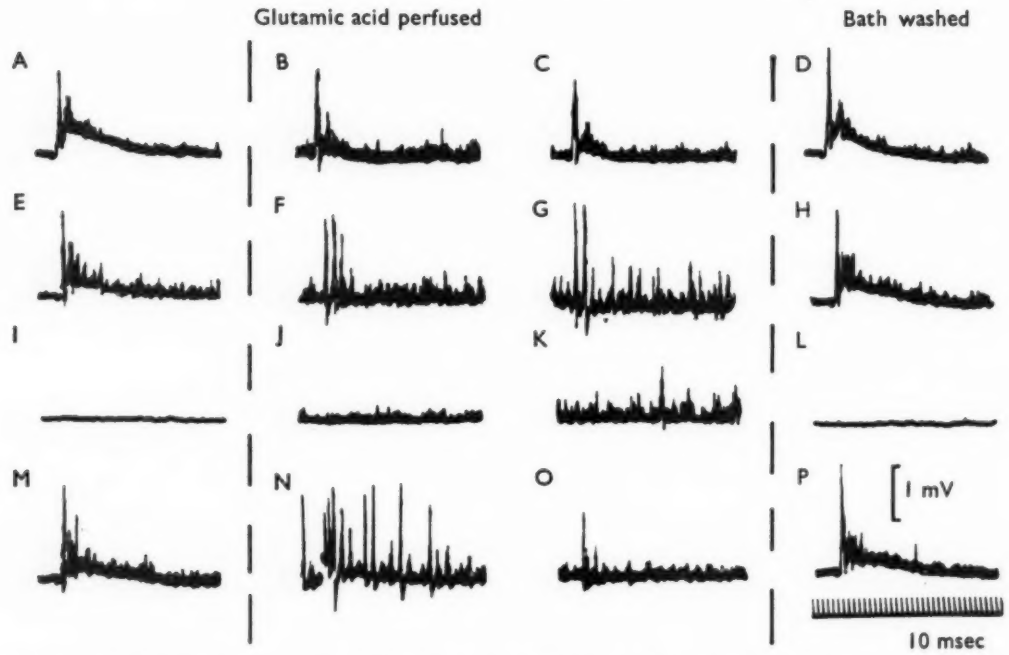


Fig. 3. The action of L-glutamic acid upon reflex activity A to H, M to P, and upon spontaneous activity recorded from the ventral root, I to L. A, E, I, and M—controls. B, F, and J were recorded 10 sec and C, G, and K 30 sec after the application of solutions containing 5×10^{-4} M, 5×10^{-3} M, and 5×10^{-2} M L-glutamic acid respectively. N and O were recorded 50 and 56 sec after the application of 10^{-2} M L-glutamic acid solution. D, H, L, and P, 120 sec after washing bath with Ringer solution. There was an interval of 15 min between each series. Time, 10 msec. Voltage calibration, 1 mV.

concentrations of glutamate in causing a progressive increase in the reflex response to a maximum, this being followed by sudden and severe depression during which dorsal root stimulation evoked practically no response from the ventral root: M is the control response, N and O were recorded 50 and 56 sec after application of 10^{-2} M L-glutamic acid; the bath was then flushed with Ringer solution and the response P was recorded 2 min later.

Action on the slow potential recorded from the ventral root following dorsal root stimulation. Although the number of spike components of the ventral root reflex was greatly increased by L-glutamic acid, the slow potential upon which these spikes were superimposed was markedly decreased. This can be seen both in the responses of Fig. 3 and those of Fig. 4. The latter figure shows, on a compressed time scale, the change in the magnitude of these slow potentials induced by three concentrations of L-glutamic acid. The responses were recorded using a direct-coupled amplifier in conjunction with a pen recorder. The left- and right-hand arrows on each of the records A to C demonstrate the points of application and washing out of the solutions respectively. The alteration in the position of the base line in these records is an indication of changes in the prevailing potential across motoneurone membranes, such potentials spreading electrotonically along the ventral roots. Fig. 4 shows the changes in the base line potential induced by 2×10^{-4} M (A), 3×10^{-4} M (B) and

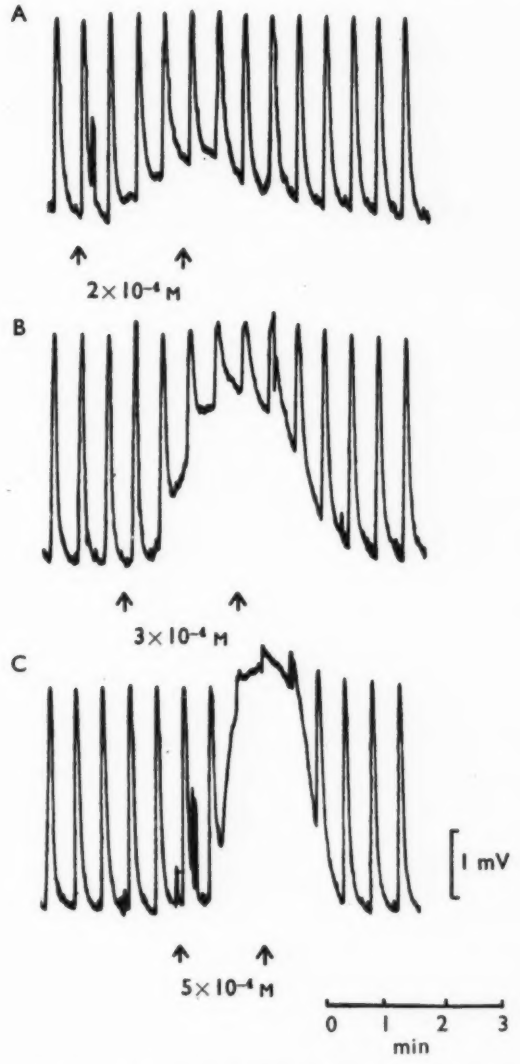


Fig. 4. Potentials recorded from the ventral root of a lumbar preparation using a direct-coupled amplifier and a paper recorder. The dorsal root was stimulated maximally twice per min throughout. At the first arrow in each record L-glutamic acid at concentrations of 2×10^{-4} M (A), 3×10^{-3} M (B), and 5×10^{-4} M (C) was perfused through the bath for 2, 2, and 1.5 min respectively. The bath was washed out at the second arrow. Time, min. Voltage calibration—1 mV for all records.

5×10^{-4} M (C) L-glutamic acid, and the concomitant progressive decreases in the magnitude of the slow potential as measured from this base line. The effect, which reflects the extent of the glutamate-induced depolarization of motoneurones, was greater the higher the concentration of the amino-acid. As can be seen from Fig. 2, γ-aminobutyric acid also caused a marked decrease in the magnitude of the slow potential, but the effect in this instance was not accompanied by any significant change in the base line potential.

Potentials produced in the ventral root by L-*glutamic acid in the absence of dorsal root stimulation.* The application of relatively dilute solutions (less than 5×10^{-4} M)

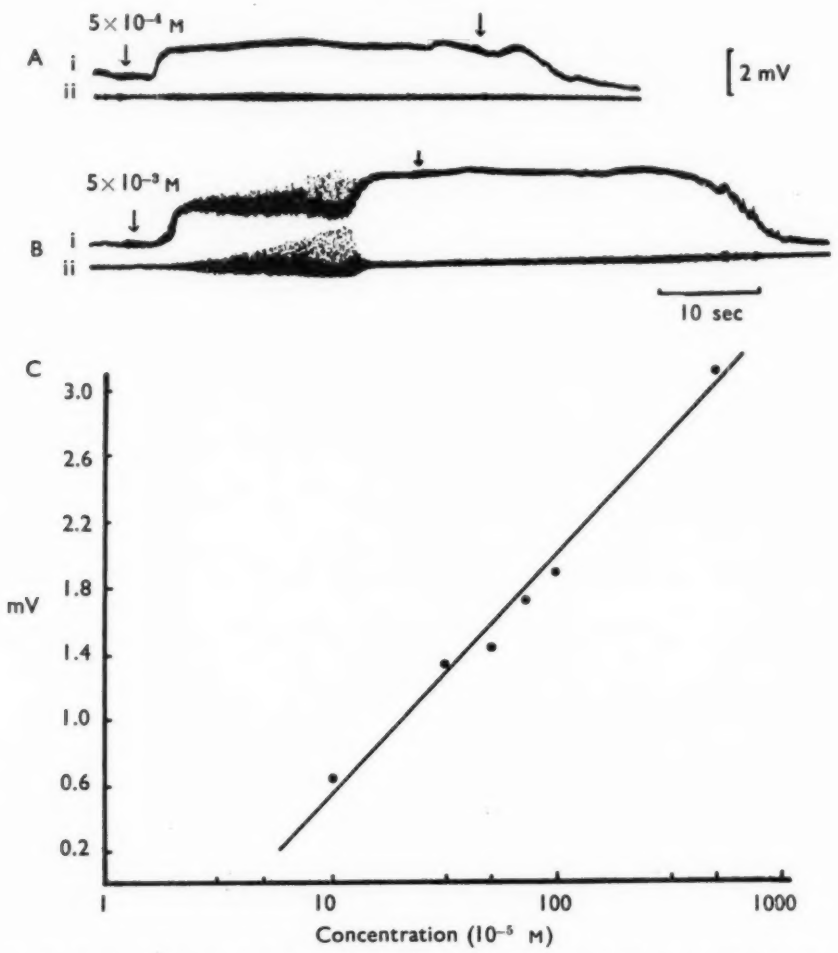


Fig. 5. A, B, Potentials recorded from the ventral root of a cervical preparation in the absence of dorsal root stimulation. Negativity upwards. i—Using a direct-coupled amplifier; ii—using an amplifier having a 5 msec time constant. A—5×10^{-4} M L-glutamic acid was perfused through the bath (first arrow) for 35 sec. The second arrow indicates the time when the bath was washed out. B—5×10^{-3} M L-glutamic acid applied in a similar fashion for 28 sec. Time 10 sec. Voltage calibration, 2 mV for i and ii. C, The magnitude of the potential recorded from the ventral root, in an experiment similar to that of A and B, in response to L-glutamic acid. Ordinate, potential in mV. Abscissae, concentration of L-glutamic acid in the perfusing solution. All applications were of 45 sec duration.

of L-glutamic acid to the isolated toad spinal cord resulted in a depolarization of motoneurones, this being recorded as a negative potential by the electrode situated on the ventral root. Higher concentrations of glutamic acid caused the development of a greater negative potential and also evoked discharges of motoneurones as indicated by spike responses recorded from the ventral root. Fig. 5 illustrates these effects; A i and B i are the traces obtained of the potentials recorded between the ventral root and the bath with a direct-coupled amplifier, and A ii and B ii are the records of spike responses recorded from the ventral root with an amplifier of short time constant (5 msec). When 5×10^{-4} M L-glutamic acid (A) was used, relatively few spike discharges were recorded from the ventral root (record A ii); however,

3 sec after the application of this solution (left-hand arrow in A) a negative potential was recorded from the ventral root (A i); this potential rapidly attained a plateau which was thereafter maintained until shortly after the glutamic acid solution was flushed out (right-hand arrow in A). Recovery took 10 to 12 sec. The higher concentration of glutamic acid used in B (5×10^{-3} M) caused many motoneurones to discharge impulses, as is evident from both records, B i and B ii. An increasing number of motoneurones became involved until a maximum was reached, at which point spike responses ceased and the depolarization ascended to a new plateau, this being maintained for approximately 25 sec after the glutamate solution was washed out. Renewed spike activity was evident during the later stages of the recovery, which required about 40 sec to reach completion. The cessation of high-frequency spike activity concomitant with the rise in the level of depolarization during the presence of glutamate has a parallel in the sudden decline in reflex responses with high concentrations of glutamate, as seen in Fig. 3, O. The type of response exemplified by the records B i and B ii of Fig. 5 could be obtained with all preparations, but the required concentration of glutamic acid varied somewhat from preparation to preparation.

The potential changes evoked by a series of increasing concentrations of L-glutamic acid are shown for a cervical preparation in Fig. 5, C, in which the logarithm of the concentration of the amino-acid is plotted against the negative potential observed. The relationship was approximately linear over the range of concentrations used. Similar results were obtained from lumbar preparations. Unlike the depressant action of γ-aminobutyric acid, the excitatory efficacy of L-glutamic acid showed no decline after repeated application in high concentrations.

Comparison of the potencies of depressant and excitant substances

Two methods were employed for obtaining a quantitative measure of the actions of substances on the isolated toad cord: for depressant substances, the extent of the reduction of the integrated ventral root response following dorsal root stimulation; and, for excitant substances, the magnitude of the negative potentials recorded from the ventral root in the absence of dorsal root stimulation. The latter could also be used qualitatively to differentiate substances whose actions resulted in a depolarization of motoneurones from those substances which did not have this excitant action. This was not so, however, for the integrated ventral root response technique, since excitants, as well as depressants, caused a reduction in the magnitude of these responses. The integrator was used only to summate potentials produced in the ventral root following a dorsal root stimulus, and did not take into account the very slow potentials induced in the ventral root by drug action. As can be seen from the records of Fig. 5, A and B, L-glutamic acid caused a potential to be recorded from the ventral root independently of dorsal root stimulation, and when stimuli were applied to the dorsal root (Fig. 4) the ventral root responses superimposed upon the glutamate-induced potential were smaller than those recorded in the absence of glutamic acid. An integrated record of these responses would have shown a marked reduction in comparison with the control responses recorded in the absence of glutamic acid. Thus, the mere reduction of the integrated record

of the ventral root response following dorsal root stimulation cannot of itself be taken to indicate depressant activity. Therefore, if this technique is to be used for comparing the potencies of purely depressant compounds, it is necessary that such a depressant action be definitely established for each substance by prior tests. All the substances to be compared were therefore first investigated for their effect on the fast components of the reflex response, excitatory and depressant actions being clearly distinguishable by this test (as shown in Figs. 3 and 1).

Depressant substances related to γ-aminobutyric acid. The substances of Table 1 bear varying degrees of structural similarity to γ-aminobutyric acid, all being neutral amino-acids or closely related thereto. Those substances which did not enhance the fast components of the reflex following dorsal root stimulation were tested for depressant potency relative to γ-aminobutyric acid. An indication of the relative potency of one compound to another was obtained by comparing the concentration of each required to produce the same diminution of the integrated ventral root response. The substances tested were so selected that they formed a series which both paralleled and supplemented similar series previously investigated under other test conditions (Purpura *et al.*, 1959 ; Edwards & Kuffler, 1959 ; Robbins, 1959 ; Van Harreveld, 1959 ; Curtis & Watkins, 1960). The results are given in Table 1, where the potencies are expressed relative to that of γ-aminobutyric acid.

The substances fall into three main categories, aminocarboxylic acids, aminosulphonic acids, and guanidinocarboxylic acids, the other substances being simply derived from members of these groups. Although belonging chemically more to the γ-aminobutyric acid series than to the glutamic acid series, the substances were not all depressant, several indeed exhibiting weak excitatory action. Discussion of these substances is deferred until after that of the excitatory amino-acids related to glutamic acid. Within the three groups A, C and D of Table 1, a similar, but not identical, pattern of activity with respect to chain length was observed. Thus, amongst the ω-aminoalkanecarboxylic acids (Table 1, A), peak depressant activity was associated with a chain length of two carbon atoms between the amino-group and the carboxyl group (β-alanine), whilst in the series of ω-aminoalkanesulphonic acids (Table 1, C), in the most active substance (3-aminopropanesulphonic acid), the functional groups are separated by a three-carbon-atom chain. This compound was the most potent depressant tested. In the ω-guanidinoalkanecarboxylic acids (Table 1, D) maximum activity occurred where only one carbon atom separated the guanidino-group and the carboxyl group (glycocyamine), but it is probably significant that again three atoms (in this case, –C–N–C–) separate the acidic group from the terminal amino-group, as was also pointed out by Purpura *et al.* (1959) and Edwards & Kuffler (1959). A particularly noticeable feature of the ω-aminoalkanesulphonic acid group (Table 1, C) was the very sharply defined optimum chain length for depressant activity, this activity falling off steeply if the chain length was less than two carbon atoms and being transformed into a weakly excitatory action if the chain length exceeded three carbon atoms. A similar transformation of depressant into excitatory action was also observed in the ω-amino- and ω-guanidinoalkanecarboxylic acids, although in these cases the change-over was less abrupt.

TABLE 1

STRUCTURE–ACTIVITY RELATIONSHIPS OF γ-AMINOBUTYRIC ACID SERIES

Column 1: The ratio of the concentration of γ-aminobutyric acid (10^{-3} M) to that of the test substance which causes the same degree of depression of the ventral root potentials evoked by dorsal root stimulation. Column 2: Approximate relative activities. (a) Depressant compounds, γ-aminobutyric acid=1·0. <0·1, −; 0·1 to 0·3, −−; 0·3 to 1·0, −−−; 1·0 to 3·0, −−−−; 3·0 to 10, −−−−−. (b) Excitant compounds, based on the method used in Table 2, see columns 1 and 2 of Table 2

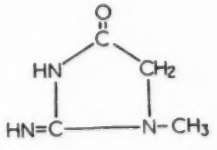
Structural feature of interest	Compound	Structure	1	2
A. Distance between carboxyl group and amino-group	Glycine	$H_2N.CH_2.CO_2H$	0·67	−−−
	β-Alanine	$H_2N.[CH_2]_2.CO_2H$	1·4	−−−−
	γ-Aminobutyric acid	$H_2N.[CH_2]_3.CO_2H$		−−−−
	δ-Aminovaleric acid	$H_2N.[CH_2]_4.CO_2H$	0·22	−−
	6-Aminohexanoic acid	$H_2N.[CH_2]_5.CO_2H$		0
	7-Aminoheptanoic acid	$H_2N.[CH_2]_6.CO_2H$		(+)
	8-Amino-octanoic acid	$H_2N.[CH_2]_7.CO_2H$		(+)
	9-Aminononanoic acid	$H_2N.[CH_2]_8.CO_2H$		+
B. *N*-Alkylation	*N*-Methyl-β-alanine	$CH_3.NH.[CH_2]_2.CO_2H$	0·25	−−
	NN-Dimethyl-β-alanine	$(CH_3)_2N.[CH_2]_2.CO_2H$	0·14	−−
	β-Propiobetaine hydrochloride	$(CH_3)_3N^+[CH_2]_2.CO_2H(Cl^-)$		0
	γ-Amino-*N*-methylbutyric acid	$CH_3.NH.[CH_2]_3.CO_2H$	0·14	−−
	γ-Amino-*NN*-dimethyl-butyric acid	$(CH_3)_2.N.(CH_2)_3.CO_2H$	0·05	−
	γ-Butyrobetaine	$(CH_3)_3N^+.[CH_2]_3.CO_2^-$		0
C. Replacement of the carboxyl group by the sulphonic acid group	Aminomethanesulphonic acid	$H_2N.CH_2.SO_3H$	0·02	−
	Taurine	$H_2N.[CH_2]_2.SO_3H$	1·0	−−−−
	3-Aminopropanesulphonic acid	$H_2N.[CH_2]_3.SO_3H$	3·3	−−−−−
	4-Aminobutanesulphonic acid	$H_2N.[CH_2]_4.SO_3H$		+
	N-Methyltaurine	$CH_3.NH.[CH_2]_2.SO_3H$		0
D. Replacement of the amino-group by the guanidino-group	Glycocyamine	$H_2N.C(:NH).NH.CH_2.CO_2H$	1·0	−−−−
	β-Guanidinopropionic acid	$H_2N.C(:NH).NH.[CH_2]_2.CO_2H$	0·17	−−
	γ-Guanidinobutyric acid	$H_2N.C(:NH).NH.[CH_2]_3.CO_2H$		0
	δ-Guanidinovaleric acid	$H_2N.C(:NH).NH.[CH_2]_4.CO_2H$		(+)
	Creatine	$H_2N.C(:NH).N(CH_3).CH_2.CO_2H$		0
E. Miscellaneous	D-α-Alanine	$CH_3.CH(NH_2).CO_2H$	0·25	−−
	L-α-Alanine		0·18	−−
	D-Serine	$HO.CH_2.CH(NH_2).CO_2H$		++
	L-Serine			(+)
	DL-α-Aminoisobutyric acid	$CH_3.C(NH_2)(CH_3).CO_2H$		0
	DL-Norvaline	$CH_3.[CH_2]_2.CH(NH_2).CO_2H$		(+)
	L-Valine	$CH_3.CH(CH_3).CH(NH_2).CO_2H$		(+)
	DL-Norleucine	$CH_3.[CH_2]_3.CH(NH_2).CO_2H$		(+)
	L-Leucine	$CH_3.CH(CH_3).CH_2.CH(NH_2).CO_2H$		(+)
	DL-Isoleucine	$CH_3.CH_2.CH(CH_3).CH(NH_2).CO_2H$		(+)
	Isethionic acid	$HO.[CH_2]_2.SO_3H$		0
	Ethanolamine	$H_2N.CH_2.CH_2.OH$		0
	β-Alaninamide	$H_2N.[CH_2]_2.CONH_2$		0
	γ-Aminobutyramide	$H_2N.[CH_2]_3.CONH_2$		0
	Creatinine			+

TABLE 2

STRUCTURE–ACTIVITY RELATIONSHIPS OF L-GLUTAMIC ACID SERIES

Column 1: The ratio of the concentration of L-glutamic acid (5×10^{-4} M) to that of the test substance which causes the same negative potential change in the ventral root. Column 2: Approximate relative activities, L-glutamic acid= 1·0. <0·05, (+); 0·05 to 0·15, +; 0·15 to 0·5, ++; 0·5 to 1·5, +++; 1·5 to 5, ++++; 5 to 15, +++++; 15 to 50, ++++++; >50, +++++++. *Mixture of diasterioisomers.

Structural feature of interest	Compound	Structure	1	2
A. Distance between the amino-group and the most distant (ω) carboxyl group	Aminomalonic acid	$HO_2C.CH(NH_2).CO_2H$	1·2	+++
	DL-Aspartic acid	$HO_2C.CH_2.CH(NH_2).CO_2H$	1·0	+++
	DL-Glutamic acid	$HO_2C.[CH_2]_2.CH(NH_2).CO_2H$	2·0	++++
	DL-α-Aminoadipic acid	$HO_2C.[CH_2]_3.CH(NH_2).CO_2H$	0·12	+
	DL-α-Aminopimelic acid	$HO_2C.[CH_2]_4.CH(NH_2).CO_2H$	0·17	++
	DL-α-Aminosebacic acid	$HO_2C.[CH_2]_7.CH(NH_2).CO_2H$		0
B. Optical configuration (see also F, H and J)	D-Aspartic acid	See A.	1·2	+++
	L-Aspartic acid		0·8	+++
	D-Glutamic acid		7·5–2·5	++++
	L-Glutamic acid			+++
C. Variation of the ω-acidic group	L-Cysteic acid	$HO_3S.CH_2.CH(NH_2).CO_2H$	0·6	+++
	DL-Homocysteic acid	$HO_3S.[CH_2]_2.CH(NH_2).CO_2H$	25	++++++
	L-α-Amino-β-sulphinopropionic acid	$HO_2S.CH_2.CH(NH_2).CO_2H$	0·8	+++
D. Relative disposition of the amino-group and the carboxyl group	β-Aminoglutaric acid	$HO_2C.CH_2.CH(NH_2).CH_2.CO_2H$	0·42	++
	2-Aminoethylmalonic acid	$HO_2C.CH(CH_2.CH_2.NH_2).CO_2H$		0
	DL-2-(Aminomethyl)-succinic acid	$HO_2C.CH_2.CH(CH_2.NH_2).CO_2H$	2·0	++++
E. Substitution in the carbon chain	β-Hydroxy-DL-glutamic acid*	$HO_2C.CH_2.CH(OH).CH(NH_2).CO_2H$	1·2	+++
	α-Methyl-DL-glutamic acid	$HO_2C.[CH_2]_2.C(CH_3)(NH_2).CO_2H$	0·05	+
	DL-αε-Diaminopimelic acid	$HO_2C.CH(NH_2).[CH_2]_3.CH(NH_2).CO_2H$		0
F. *N*-Alkylation	*N*-Methyl-DL-aspartic acid	$HO_2C.CH_2.CH(NH.CH_3).CO_2H$	25	++++++
	N-Methyl-D-aspartic acid		70	+++++++
	N-Methyl-L-aspartic acid		5·0	+++++
	NN-Dimethyl-DL-aspartic acid	$HO_2C.CH_2.CH\{N(CH_3)_2\}.CO_2H$	0·5	+++
	N-Methyl-DL-glutamic acid	$HO_2C.[CH_2]_2.CH(NH.CH_3).CO_2H$	3·8	++++
	NN-Dimethyl-L-glutamic acid	$HO_2C.[CH_2]_2.CH\{N(CH_3)_2\}.CO_2H$	0·25	++
	α-Glutarobetaine	$HO_2C.[CH_2]_2.CH\{N^+(CH_3)_3\}.CO_2^-$		0
G. Replacement of the amino-group by the formamidino group	*N*-Iminomethyl-L-aspartic acid	$HO_2C.CH_2.CH(NHCH=NH).CO_2H$	0·63	+++
H. ω-Amide linkages	D-Asparagine	$HO_2C.CH(NH_2).CH_2.CONH_2$	1·2	+++
	L-Asparagine		0·33	++
	N-Methyl-DL-asparagine	$HO_2C.CH(NH.CH_3).CH_2.CONH_2$	0·1	+
	β-Sulphamoyl-L-alanine	$HO_2C.CH(NH_2).CH_2.SO_2NH_2$	0·071	+
	L-Glutamine	$HO_2C.CH(NH_2).[CH_2]_2.CONH_2$	0·025	(+)
I. α-Amide linkages	DL-Isoasparagine	$HO_2C.CH_2.CH(NH_2).CONH_2$		0
	L-Isoglutamine	$HO_2C.[CH_2]_2.CH(NH_2).CONH_2$		0
J. Replacement of the amino-group by the hydroxyl group	Tartronic acid	$HO_2C.CH(OH).CO_2H$	0·05	+
	D-Malic acid	$HO_2C.CH_2.CH(OH).CO_2H$	0·5	+++
	L-Malic acid		0·05	+

N-Alkylation (Table 1, B) caused a graded reduction of depressant activity as the extent of the alkylation increased. This was true both for β-alanine and for γ-aminobutyric acid series, in which the trimethyl derivatives were inactive, the dimethyl compounds were slightly active, and the monomethyl derivatives, although more active, were still but weak depressants compared with the unmethylated parent compounds. A single *N*-methyl substituent completely abolished the depressant activity of taurine (compare, for example, taurine and *N*-methyltaurine).

Amongst the series of compounds having miscellaneous structural relationships with γ-aminobutyric acid (Table 1, E) are several α-amino-acids which were tested primarily because of the strong depressant activity of glycine. Of these, α-alanine was the only one to show depressant activity, the D form of this compound being slightly stronger than the L form. Other α-amino-acids, and also creatinine, were found to have excitatory actions, all of which, however, were extremely weak except that of D-serine, which was a moderately strong excitant. The other compounds in this section were inactive.

Excitatory substances related to glutamic acid. The substances of Table 2 bear varying degrees of structural similarity to L-glutamic acid, all being acidic amino-acids or closely related thereto. Their excitant potencies relative to L-glutamic acid were estimated on the basis of the concentrations of each required to produce the same negative potential in the ventral root. The structure–activity relationships apparent from the results listed in Table 2 show, with some exceptions, a very close correlation with those observed amongst the depressant compounds of Table 1. Thus, in the aminodicarboxylic acid group (Table 2, A), optimum chain length separating the amino-group and ω-carboxyl group proved to be three carbon atoms; in the depressant series (Table 1, A) the optimum chain length separating the analogous groups was two carbon atoms. Replacement of the ω-carboxyl group of the most active members of the aminodicarboxylic acid series by sulphonic or sulphinic acid groups leads to the compounds of Table 2, C, which were found to be very active excitants. Indeed, DL-homocysteic acid proved to be 25 times stronger than L-glutamic acid and was one of the most active excitants tested. This correlates with the ob ervation that 3-aminopropanesulphonic acid (Table 1, C) was the most potent depressant. Further correlation is seen in the relative effects of D and L forms of depressant and excitant compounds, D forms being the stronger in every instance (Table 1, E, and Table 2, B, F, H, J).

Less correlation between the depressant and excitant series is observed with *N*-methylation (Table 1, B, and Table 2, F). Whereas mono-*N*-methyl derivatives of β-alanine and γ-aminobutyric acid were weaker depressants than the parent compounds, the mono-*N*-methyl derivatives of aspartic and glutamic acids were very much stronger excitants than their unmethylated counterparts. Thus, *N*-methyl-DL-glutamic acid (Table 2, F) and *N*-methyl-DL-aspartic acid (Table 2, F) were found to be respectively 2 and 25 times stronger than the parent compounds. The D isomer of the latter substance was the strongest excitant found, and was 14 times stronger than the L isomer and 70 times stronger than the reference compound, L-glutamic acid. The di- and tri-*N*-methyl derivatives of glutamic acid and the

di-*N*-methyl derivative of aspartic acid were all much less active than the unmethylated, parent compounds, which again parallels the findings within the depressant series.

Another exception to the otherwise close parallelism between the respective structure–activity relationships of the two series is afforded by asparagine (Table 2, H). This compound, particularly the D form, displayed marked excitatory activity which is in contrast to the inactivity of β-alaninamide (Table 1, E), the analogous compound in Table 1. The ω-amides of L-glutamic acid (L-glutamine, Table 2, H) and L-cysteic acid (β-sulphamoyl-L-alanine, Table 2, H) also proved to be excitants, but each was considerably weaker than L-asparagine. In contrast to the ω-amides, the α-amides of aspartic and glutamic acids (DL-isoasparagine and L-isoglutamine, respectively, Table 2, I) were inactive.

The remaining sections of Table 2 demonstrate the effects of several other types of structural modification in the excitant series. Isomers of glutamic acid (Table 2, D), arising from changes in the relative positions of the amino-group and the two carboxyl groups, varied in potency, the most active being the branched-chain compound, DL-2-(aminomethyl)succinic acid. Substituents in the carbon chain linking the two carboxyl groups of glutamic acid (Table 2, E) reduced activity, this being very marked with an α-methyl group (α-methyl-DL-glutamic acid), but much less so with a β-hydroxyl group (β-hydroxy-DL-glutamic acid). Conversion of the amino-group of L-aspartic acid into the formamidino-group gives the compound *N*-iminomethyl-L-aspartic acid (Table 2, G), which was found to be only slightly less active than the parent compound. Replacement of the amino-groups of aspartic and aminomalonic acids by hydroxyl groups leads respectively to malic and tartronic acids (Table 2, J), each of which exhibited excitatory activity. This was quite marked in D-malic acid but weak in L-malic and tartronic acids.

Other excitatory amino-acids. Preliminary testing of the substances of Table 1 on the fast components of the reflexes recorded from the ventral root in response to dorsal root stimulation demonstrated that several of the substances had excitatory actions. Amongst these were ω-amino- and ω-guanidino-alkanecarboxylic acids of chain length greater than that at which depressant activity reached a minimum during the ascent of a homologous series (Table 1, A and D). A similar situation occurred in the ω-aminoalkanesulphonic acid series (Table 1, C), but, in these, excitatory activity in the series appeared immediately following the peak of depressant activity. The concentrations of these substances necessary to produce significant increases in the reflex responses were mostly of the order of 10^{-2} M or greater, and such compounds must therefore be classed as only very weak excitants. The excitatory amino-acids of Table 1, like those of Table 2, also caused a negative potential which was recorded by an electrode on the ventral root, but in general the time courses of such actions were somewhat different from those observed with substances of the glutamic acid type. With the relatively low concentrations used, the potentials caused by the latter group developed rapidly after application of the substance, thereafter maintaining a steady level until the substance was washed out. Recovery was also rapid. However, in the long-chain ω-amino, ω-guanidino- and α-amino-alkane acids, the negative potentials developed only slowly, and recovery

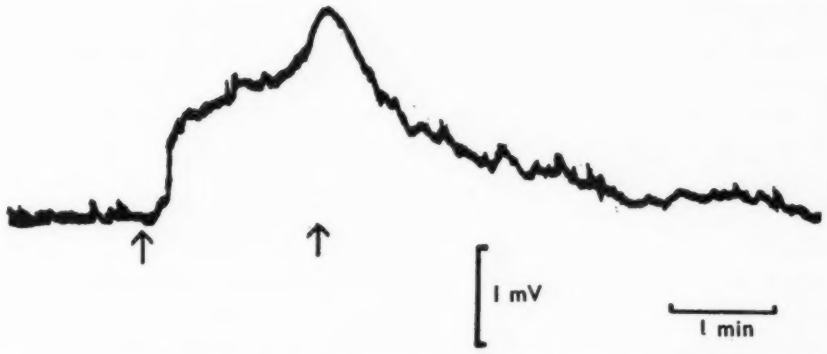


Fig. 6. The potential recorded from the ventral root of a lumbar preparation in response to perfusing the bath with a solution containing 10^{-2} M 9-aminononanoic acid. The amino-acid was applied for 90 sec as indicated by the two arrows. Time, min. Voltage calibration, 1 mV, negativity upwards.

was similarly protracted. A typical example is provided by 9-aminononanoic acid, illustrated in Fig. 6. Approximately 10 sec after the application of 10^{-2} M 9-aminononanoic acid, a negative potential was recorded from the ventral root, and this increased irregularly until the bath was flushed 90 sec later. Recovery was slow and was still incomplete after 5 min.

Because of these rather different time courses, it was difficult to compare accurately the excitatory amino-acids of this type with those of the glutamate type in regard to their respective potencies. The symbolic indications of their relative potencies given in Table 1 were based upon the approximate concentrations of each which were necessary to produce within two or three minutes the same potential as that produced by 5×10^{-4} M L-glutamate in 45 sec. D-Serine (Table 1, E) acted somewhat more rapidly than did the rest of these substances, and was the most active of the group, being approximately one-half as strong as L-glutamic acid. The L isomer (Table 1, E) was very much weaker. 9-Aminononanoic acid (Table 1, A) and 4-aminobutanesulphonic acid (Table 1, C) were the next strongest, these substances both having about one-twentieth of the excitatory activity of L-glutamic acid.

DISCUSSION

The present studies were undertaken for the purpose of investigating the possible use of the isolated hemisected spinal cord of the toad in a screening programme for substances having an action upon neurones. Such a preparation offers the advantages that it is readily set up, the tissue usually remains responsive to electrical stimulation for many hours, and substances can be applied directly and in controlled concentrations (Bunzl, Burgen, Burns, Pedley & Terroux, 1954 ; Angelucci, 1956). The technique also avoids some of the uncertainties inherent in many other test situations, since effects observed cannot rise indirectly from peripheral receptors or follow alterations in blood flow (Curtis, Phillis & Watkins, 1961). Furthermore, the access of the applied substances to the centrally located neurones cannot be hindered by the blood–brain barrier. However, any observed action involves possible effects upon many cells, some types of which may react in a qualitatively or quantitatively

different manner from other types. This feature precludes direct knowledge of the precise site and mode of action of a particular test substance, but may occasionally be an advantage by making apparent actions of substances which may not be detected by more restrictive methods. For the purposes of the present study, two series of amino-acids and substances closely related to amino-acids were selected so as to cover the most important findings of several similar investigations undertaken recently on a variety of excitable tissues (Purpura *et al.*, 1959 ; Edwards & Kuffler, 1959 ; Robbins, 1959 ; Van Harreveld, 1959 ; Curtis & Watkins, 1960). γ-Aminobutyric acid and L-glutamic acid were chosen as standard depressant and excitant substances respectively, and their actions upon the responses of the isolated hemisected toad cord were briefly investigated prior to their use as reference compounds for the estimation of the relative potencies of the other substances in the two series.

γ-Aminobutyric acid was selected as the standard depressant substance for the reasons that on all preparations it appears to be amongst the most potent of the ω-amino-acids and its action has been the most widely studied. Thus, γ-aminobutyric acid depresses the responses of the amphibian spinal cord *in situ* (Sigg & Grundfest, 1958 ; Tsujioka & Fukuya, 1958), the stretch receptor of the crayfish (Bazemore, Elliott & Florey, 1956 ; Edwards & Kuffler, 1957 ; Hagiwara, Kusano & Saito, 1960), the perfused lobster heart (Enger & Burgen, 1957), the lobster cardiac ganglion (Maynard, 1958), mammalian ileum (Hobbiger, 1958), mammalian cerebral cortex (Hayashi & Nagai, 1956 ; Purpura, Girado & Grundfest, 1957 ; Iwama & Jasper, 1957 ; Jasper, Gonzales & Elliott, 1958 ; Marrazzi, Hart & Rodriguez, 1958 ; Takahashi, Nagashima, Koshino & Takahashi, 1959), and mammalian spinal neurones (Curtis, Phillis & Watkins, 1959). The observed depressant action of γ-aminobutyric acid on the reflex responses of the isolated hemisected spinal cord of the toad is in accordance with the observations of Sigg & Grundfest (1958) and Tsujioka & Fukuya (1958) on responses recorded *in situ* from the spinal cord of the bull frog.

L-Glutamic acid was selected as the standard excitatory substance for similar reasons to those relating to the choice of γ-aminobutyric acid as the standard depressant, and also because of its close structural relationship with the latter. Glutamic acid has been shown to have an excitant action on crustacean muscle (Robbins, 1959 ; Van Harreveld, 1959 ; Van Harreveld & Mendelson, 1959) and mammalian spinal neurones (Curtis, Phillis & Watkins, 1960). The action of this substance in eliciting spreading depression of mammalian cortical cells (Van Harreveld, 1959 ; Purpura *et al.*, 1959) also probably results from a preliminary excitatory action, followed, in this case, by excessive depolarization which inactivates the cells (Curtis & Watkins, 1960). In the case of the isolated toad cord, L-glutamic acid was found to cause a marked enhancement of the fast components of the reflex evoked by dorsal root stimulation. With high glutamic acid concentrations, this increased excitation reached a maximum and then rapidly subsided, presumably because excessive depolarization led to inactivation of the spike mechanism of many of the cells (Curtis, Phillis & Watkins, 1960). During the initial period of enhanced excitability, spike responses were evoked independently of dorsal root stimulation. The depolarizations of motoneurones responsible for these effects were propagated

electrotonically along axons of the ventral root where they were recorded as negative potentials in relation to an indifferent electrode situated in the bath. The extent of the depolarization was related to the concentration of the amino-acid. It is significant that the potentials recorded in cases of high glutamic acid concentration showed a sharp increase at the point where high-frequency spike activity ceased, this fact supporting the postulate that such cessation is associated with excessive depolarization of previously discharging cells. Following the action of high concentrations of glutamic acid, spike activity, which had ceased during the continued presence of the amino-acid, transiently recommenced during the period following washing. This effect was probably due to partial reactivation of the spike generating mechanism as the glutamate-induced depolarization subsided towards the threshold for spike initiation.

In the investigation of the relative activities of substances related to γ-aminobutyric acid and glutamic acid the most interesting result was the greater potency of the D compared with the L forms of active substances in both series. This was found to be true of every substance where both isomers were available. The stereo-difference was more marked, as would be expected, the larger the difference in the sizes of the groups attached to the asymmetric carbon atom. Thus, there was a fourteen-fold difference between the D and L forms of *N*-methylaspartic acid, but only a very slight difference in the two forms of aspartic acid. *N*-Methyl-D-aspartic acid was indeed a remarkably potent excitant, being 70 times more effective than L-glutamic acid on a concentration basis. This correlates to some extent with the fact that the DL form of the same compound had a very prolonged action when iontophoretically applied to interneurones in the cat's spinal cord (Curtis & Watkins, 1960), but in this case the compound was rated somewhat weaker than L-glutamic acid because the latter required smaller iontophoretic currents to produce similar frequencies of interneuronal discharge. Similar, though less marked, stereo-differences were evident in the depressant actions of D- and L-α-alanine and in the excitant actions of D- and L-glutamic acid, D- and L-asparagine, D- and L-malic acid, and D- and L-serine. That a similar stereo-dependence may occur in the case of amino-acid actions on mammalian cortical cells is suggested by the greater apparent potency of D- than L-glutamic acid in producing spreading depression, although in these acids enzymic destruction of the L form has been proposed as a possible explanation (Van Harreveld, 1959). Similar considerations may also apply in the case of the toad cord results with those amino-acids whose L forms are probably normal constituents of the tissue, but would seem less likely to explain the differential potency of D and L forms of *N*-methylaspartic acid. This is a synthetic amino-acid which has not been reported to occur in nature, and neither of the forms would be expected to be metabolized at a rate sufficiently rapid to influence the results.

Another interesting result was the extremely strong actions of the sulphonic acids, 3-aminopropanesulphonic acid and DL-homocysteic acid. The former compound was found to be the most powerful depressant tested, and the latter, together with *N*-methyl-DL-aspartic acid, the strongest of all racemic excitatory compounds. The structural requirements for high activity in the aminosulphonic acid series, at least in the case of the depressants, appear to be more rigid than those for the amino-

carboxylic acids. A primary amino-group and a chain length of two or three carbon atoms between the acidic and basic terminal groups were much more essential for high depressant activity in the sulphonic acid than in the carboxylic acid series.

It is noteworthy that 3-aminopropanesulphonic acid and homocysteic acid bear the same structural relationship to each other as do γ-aminobutyric acid and glutamic acid, which provides another example of the general rule (Curtis & Watkins, 1960) that, if an ω-amino-acid is a strong depressant, the corresponding acidic amino-acid, containing an additional carboxyl group *alpha* to the amino-group, will be a strong excitant. The converse of this rule, though found to be equally true in the case of iontophoretic experiments on interneurones of the cat's spinal cord, is not valid in some instances in the case of the isolated toad cord. Thus, the strong excitant asparagine, on removal of the α-carboxyl group, becomes not a depressant but an inactive compound (β-alaninamide). The weak excitant, L-glutamine, and the inactive decarboxylation product, γ-aminobutyramide, provide an additional, if less significant, example. The corresponding amide of L-cysteic acid, β-sulphamoyl-L-alanine, also had weak excitatory activity, but the decarboxylated analogue was not available for testing.

The structure–activity relationships apparent from the results shown in Tables 1 and 2 are in general agreement with those of similar studies on the actions of the same and related substances on other tissues (Edwards & Kuffler, 1959 ; Purpura *et al.*, 1959 ; Robbins, 1959 ; Van Harreveld, 1959 ; Curtis & Watkins, 1960). Where tissues involving a multiplicity of cells were used (Purpura *et al.*, 1959 ; Van Harreveld, 1959), the correlation is extremely close. However, when comparison is made of the toad cord results with those of the same substances applied iontophoretically to single cells of the cat's spinal cord (Curtis & Watkins, 1960), certain discrepancies are apparent. These mainly involve the moderate to strong actions observed on the toad cord by D- and L-asparagine, D-serine and glycocyamine. These substances were inactive when applied iontophoretically around interneurones of the cat's spinal cord, although adequate concentrations should have been obtained to affect the excitability of these cells if the substances were indeed active thereon. The lack of correlation between these two sets of results may possibly be extended to include the long-chain ω-amino- and ω-guanidino-alkane acids, as well as several other substances which were found to be weak excitants on the frog cord. These substances also were without effect on single cells of the cat cord, but the concentrations attainable by the iontophoretic technique (Curtis, Perrin & Watkins, 1960) may not have been adequate to demonstrate weak actions.

It must be re-emphasized that the gross responses of a tissue composed of a mass of cells, involving almost certainly many different functional types, does not allow conclusions to be drawn regarding the sites or mechanism of action of a particular substance. Thus, the explanation of the differences observed between the gross actions of compounds on such tissues and those on single cells in other tissues may lie in a differential responsiveness of individual cells to the substances under test ; or in a possible alteration of extracellular levels of endogenous active substances because of the inhibition of enzyme systems by the test substances ; or, again, in enzymic conversion of the test substances, themselves perhaps inactive, into active

substances (Curtis & Watkins, 1960; Curtis, 1961). Indeed, a possible indication of different sites and/or modes of action of the glutamic acid and long-chain ω-aminoalkane (or ω-guanidinoalkane) acid types of excitant is the different time courses of the respective actions of these groups. The disparity in chemical structure between the two types may also be taken to indicate that different receptors and modes of actions are involved. However, this is less likely to be the case in regard to the actions of aspartic acid, asparagine, malic acid and serine, which are chemically much more closely related to one another. Furthermore, the time courses of action of these substances are not dissimilar, and the relative rapidity of these actions suggests that each is acting directly upon cell membranes. Were this to be the case, and a common site of action to be involved, certain implications with regard to the structural features of this common receptor site merit brief discussion. At physiological *p*H, asparagine, malic acid and serine do not possess the two anionic groups and one cationic group considered to be essential for combination with the amino-acid receptor of mammalian spinal neurones (Curtis & Watkins, 1960). Each of these substances does possess, however, in common with aspartic acid, three groups capable of forming co-ordinate bonds with metals. It is possible to envisage a type of receptor which could be involved in complex formation with each of these four substances, but which does not differ significantly in its main features from that proposed for mammalian spinal neurones. Thus, the receptor may involve one or two metal atoms, these being complexed with the membrane in a region to which access is limited by the nature and spatial arrangement of surrounding membrane atoms. If only one metal atom were involved, excitation could be envisaged to occur when an anionic group of the amino-acid or hydroxy acid became bound to a non-metallo-cationic site on the receptor in such a manner as to allow the two other functional groups to co-ordinate with the near-by metal atom. If two metal atoms were involved, co-ordinate bonds could form between each of the three functional groups of the substances and one or other of the metal atoms. Membrane elements in the immediate environment of the metal atoms might vary from cell to cell and tissue to tissue, and this could provide an explanation for the somewhat different structure–activity relationships which have been observed in the various investigations. These differences could thus arise because of varying degrees of interference by projecting atoms or groups of the membrane to combination of the test substance with the binding sites of the receptor. The well-known importance of calcium and magnesium in neural function (Shanes, 1958) and the occurrence of copper proteins in brain (Porter & Folch, 1957; Porter & Ainsworth, 1959) suggest possible metals which may well form complexes with neuronal membranes and which, under favourable conditions, could also form co-ordinate bonds with amino-acids and hydroxy acids.

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UPTAKE OF 5-HYDROXYTRYPTAMINE BY PLATELETS

BY

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Human blood platelets were incubated with 5-hydroxytryptamine and with tryptamine and the uptake of each amine measured. The uptake of tryptamine, unlike that of 5-hydroxytryptamine, was linearly related to the concentration of the amine in the surrounding fluid, was similar in amount at 0° and 37° C and varied directly with the *p*H of the solution. When both amines were present together the uptake of 5-hydroxytryptamine was depressed. The antagonism of tryptamine was found to be competitive, and the possible site of its action is discussed. The effect of a number of other substances on the uptake of 5-hydroxytryptamine by platelets was examined; of these imipramine, cocaine and chlorpromazine were more potent and dihydroergotamine and lysergic acid diethylamide somewhat less potent than tryptamine in inhibiting uptake.

Blood platelets incubated at 37° C with a solution of 5-hydroxytryptamine take up the amine so that its concentration in the platelets may reach a value several hundred times that of the surrounding solution. The rate of uptake has been shown to be very sensitive to temperature (Hardisty & Stacey, 1955); it is reduced by loss of carbon dioxide from the plasma in which the platelets are suspended (Born & Gillson, 1959), by the presence of various metabolic inhibitors and other substances (Born & Gillson, 1959; Sano, Kakimoto & Taniguchi, 1958; Weissbach & Redfield, 1960), and by pretreatment of the animal with reserpine (Hardisty, Ingram & Stacey, 1956). In human subjects it is diminished in certain diseases of the bone marrow (Hardisty & Stacey, 1957). Some further observations on factors affecting this mechanism are now reported.

METHODS

Platelet-rich plasma was used for these experiments, since the separation and re-suspension of human platelets impair their ability to absorb 5-hydroxytryptamine. Further, Waller, Löhr, Grignani & Gross (1959) have shown that after washing there is a marked reduction in the activity of platelet enzymes. In most of the experiments platelet-rich plasma was prepared from human blood and in a few from dog blood. All glassware with which platelets came in contact was siliconed (I.C.I. silicone fluid M441). Blood was obtained by venepuncture and collected in a tube containing a solution of disodium edetate (1 g/100 ml. 0.7% w/v sodium chloride solution) sufficient to give a final concentration of 1 mg edetate/ml. blood. The blood was cooled in ice and centrifuged at 4° C at 120 *g*, and the platelet-rich plasma pipetted off and pooled after successive periods of 10 to 15 min centrifugation. It was kept at 4° C until used; this was never more than 1 hr after the blood was drawn.

Platelet counts were made in a haemocytometer chamber using a solution of ammonium oxalate (1 g $(COO.NH_4)_2.2H_2O$/100 ml. water) as diluent. The packed platelet volume was determined by centrifuging platelet-rich plasma in the thrombocytocrit described by Hardisty & Stacey (1955) for 90 min at 2,500 *g*.

Absorption of 5-hydroxytryptamine by platelets. Two methods were used. In most experiments 0.5 to 2 ml. platelet-rich plasma was put into a 15 ml. conical centrifuge tube closed by a vaccine cap through which two needles were thrust and gassed with oxygen containing 5% carbon dioxide. The tube was held in a water bath at 37° C in a nearly horizontal position so as to expose a large surface to the gas, and rocked gently to and fro mechanically. Twelve tubes could be incubated simultaneously. After 10 min incubation a solution of 5-hydroxytryptamine in 0.9% sodium chloride solution (0.2 ml. for each 1 ml. platelet-rich plasma) was added by syringe and needle. The concentration of this solution depended on the purpose of the experiment and is given below. After the incubation period the tubes were cooled in ice and the gassing discontinued. They were centrifuged at 2,500 *g* for 30 min at 4° C, the vaccine caps removed, the supernatant poured off from the platelet pellet and the tubes left to drain for a few min, adhering drops of plasma being removed with filter paper.

In other experiments platelet-rich plasma was diluted with 9 vol. Krebs Ringer phosphate solution (Umbreit, Burris & Stauffer, 1945) from which the calcium and magnesium salts had been omitted but which contained 0.2 g glucose/100 ml. A large volume of this modified Krebs solution was needed to prevent change in *p*H during the experiment. Incubation was carried out in 25 or 50 ml. conical flasks, closed with rubber bungs to prevent evaporation, but not gassed. A solution of 5-hydroxytryptamine was added to give a final concentration of 1.5 μg/ml. After incubation the flasks were cooled in ice and an aliquot of the solution centrifuged as described above. Whether the plasma was gassed with 5% carbon dioxide or diluted with phosphate buffer, the *p*H at the end of incubation was always between 7.38 and 7.50.

Estimation of 5-hydroxytryptamine uptake

Fluorimetric measurement. The platelet pellet obtained above was mixed thoroughly with 0.5 ml. water and the concentration of 5-hydroxytryptamine in it estimated by the method of Bogdanski, Pletscher, Brodie & Udenfriend (1956). The difference between the concentration in platelets which had and which had not been incubated was then calculated.

Radioactivity measurement. In these experiments 5-hydroxytryptamine creatinine sulphate labelled with ^{14}C at the β-carbon atom and having an activity of 6.87 mc/mM was used (Radiochemical Centre, Amersham). After the platelets had been spun down they were suspended in 0.7 ml. water, frozen and thawed twice to liberate 5-hydroxytryptamine, centrifuged, 0.2 ml. of the clear supernatant added to 2.5 ml. liquid scintillator (Nuclear Enterprises 220) and the radioactivity estimated in a scintillation counter (Nuclear Enterprises No. 5503). In some of the earlier experiments 0.2 ml. supernatant was spread on 1 in. aluminium planchettes and counted in a gas-flow proportional counter. Results of the fluorimetric and radioactive methods are compared below.

Since the platelets were not washed after centrifuging, 5-hydroxytryptamine trapped in the interstices of the platelet pellet was included in the estimation. Born & Bricknell (1959) estimated the volume of trapped solution to be 0.352 μl./10^8 platelets, which was in reasonable agreement with the rough estimate made by Hardisty & Stacey (1955) using Evans blue. In most experiments the concentration of 5-hydroxytryptamine in the platelet was so great compared with that in the solution that the correction for this additional 5-hydroxytryptamine was negligible ; however, in those experiments in which high concentrations of 5-hydroxytryptamine were used and in the experiments with tryptamine the estimated concentration of absorbed amine was reduced by the calculated amount in the trapped solution estimated as 0.35 μl./10^8 platelets or 0.25 μl./μl. platelet substance.

Protein estimations were made by the method of Greenberg (1929). The *p*H determinations were all made with a glass electrode. Weights of amines refer to the free bases.

RESULTS

Effect of p*H on the uptake of 5-hydroxytryptamine.* The uptake of 5-hydroxytryptamine was determined by estimating fluorimetrically the amount in the platelets initially and after incubation at 37° C for 90 min with 1.4×10^{-5} M 5-hydroxytryptamine. In Fig. 1 the results of three experiments on platelets obtained from the same individual are shown. In two of these, platelet-rich plasma was gassed

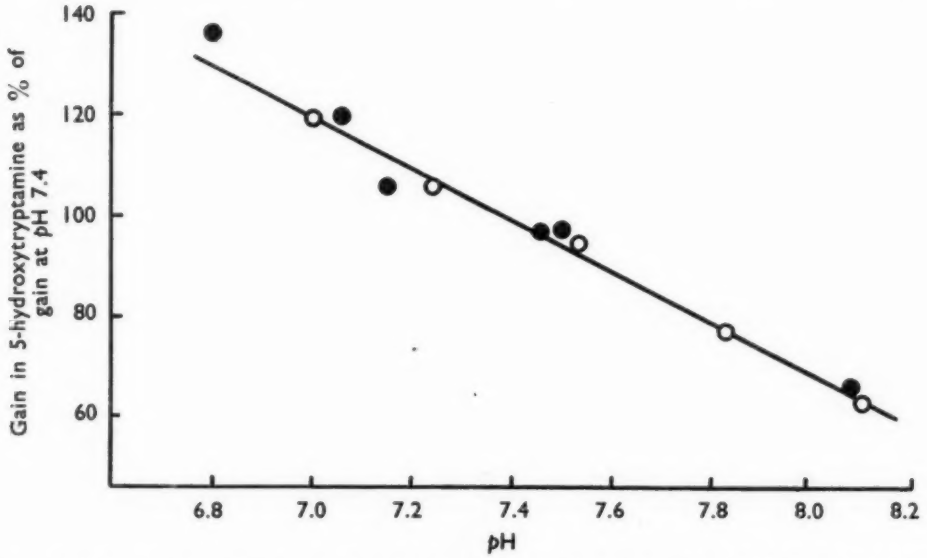

Fig. 1. Gain in 5-hydroxytryptamine by platelets after 90 min incubation at 37° C with $1{\cdot}4 \times 10^{-5}$ M 5-hydroxytryptamine at different *p*H's. In two experiments (●) *p*H was adjusted with carbon dioxide; in one experiment (○) *p*H was adjusted by adding hydrochloric acid or sodium hydroxide to the Krebs solution. Ordinate: Gain of 5-hydroxytryptamine as a % of gain at *p*H 7·4. Abscissa: *p*H of incubation mixture at the end of the experiment.

with oxygen or with oxygen containing various proportions of carbon dioxide up to 30% ; in one experiment, plasma was diluted with Krebs solution the *p*H of which had been adjusted to 5 different values by the addition of hydrochloric acid or sodium hydroxide before the addition of the platelet-rich plasma. In all cases the *p*H of the solution was determined again at the end of the experiment, and it is these values which are given in Fig. 1. The gain in 5-hydroxytryptamine is plotted as a % of the gain at *p*H 7.4 and is seen to vary inversely with the *p*H ; thus the uptake at *p*H 6.8 is nearly double that at *p*H 8.0. The results were similar whether phosphate buffer or carbon dioxide was used, but uptake was often rather less with phosphate. In an experiment with dog platelets the slope of the line was the same.

Variation in uptake with *p*H might occur if 5-hydroxytryptamine were bound to plasma protein to a degree varying with *p*H. Evidence of this was sought by dialysing plasma against buffered solutions of 5-hydroxytryptamine. Dialysis sacs made from " Visking " cellulose ester tubing and containing platelet-free plasma were suspended in solutions of 5-hydroxytryptamine in Krebs solution the *p*H of which had been previously adjusted to values ranging from 6.5 to 8.5. The con-

centration of 5-hydroxytryptamine and of protein inside and outside the sac was subsequently estimated and the *p*H of the solution inside the sac determined. The results of such an experiment in which dog plasma was dialysed against 2×10^{-5} M solutions of 5-hydroxytryptamine for 60 hr at 4° C are given in Table 1. The ratio of the concentration of 5-hydroxytryptamine inside to that outside the sac did not appear to be dependent on the *p*H of the plasma in any experiment.

TABLE 1

DISTRIBUTION OF 5-HYDROXYTRYPTAMINE BETWEEN PLASMA (INSIDE SAC) AND KREBS SOLUTION (OUTSIDE SAC) IN A DIALYSIS EXPERIMENT

	5-Hydroxytryptamine (μg/ml.)			Protein (mg/ml.)	
*p*H of plasma	Inside sac (a)	Outside sac (b)	Ratio (a)/(b)	Inside sac	Outside sac
6·6	4·23	2·93	1·4	69	0·5
7·3	4·00	3·04	1·3	71	0·5
8·3	4·12	3·02	1·4	72	0·5

Comparison of fluorimetric and radioactive tracer measurements of uptake. Platelet-rich plasma gassed with 5% carbon dioxide and containing 1.3×10^{-5} M ^{14}C-labelled 5-hydroxytryptamine was incubated for 20, 40 and 60 min. The amount of 5-hydroxytryptamine in the platelets was estimated fluorimetrically before and after incubation and by radioactivity measurements. The uptake was calculated and found to be similar by the two methods. The results of an experiment are given in Table 2.

TABLE 2

UPTAKE OF 5-HYDROXYTRYPTAMINE BY PLATELETS AFTER INCUBATION WITH ^{14}C-LABELLED 5-HYDROXYTRYPTAMINE ($1{\cdot}3 \times 10^{-5}$ M) FOR VARIOUS PERIODS

Comparison of fluorimetric and radioactivity estimation

Method of estimation	Uptake of 5-hydroxytryptamine (ng/10^8 platelets)		
	20 min	40 min	60 min
Fluorimetry	169	227	259
Radioactivity	175	237	266

The effect of oxygen, nitrogen and glucose on uptake of 5-hydroxytryptamine. Platelet-rich plasma was incubated in closed flasks with ^{14}C-labelled 5-hydroxytryptamine in Krebs solution either with glucose (0.2 g/l.) or without glucose, through which either oxygen or nitrogen had been bubbled previously for 15 min. Aerobic or anaerobic conditions were maintained during incubation by the passage of either oxygen or nitrogen through the incubation flask. The results of two experiments are given in Table 3. Uptake was unaffected by oxygen lack or by glucose lack either separately or together.

The uptake of tryptamine by platelets. Varying amounts of ^{14}C-labelled tryptamine were added to platelet-rich plasma gassed with 5% carbon dioxide in oxygen and shaken gently either at 0° or at 37° C for 30 min. The radioactivity in the platelets was then estimated. The results of such an experiment are shown

TABLE 3
EFFECT OF GLUCOSE AND OXYGEN ON THE UPTAKE OF 5-HYDROXYTRYPTAMINE BY PLATELETS

Conditions	Glucose added (mg/ml.)	Uptake of ^{14}C-labelled 5-hydroxytryptamine (μg/ml. platelet substance) Experiment 1	Experiment 2
Aerobic	2	185	170
Aerobic	0	181	176
Anaerobic	2	192	181
Anaerobic	0	182	179

in Fig. 2. The uptake of tryptamine is seen to vary linearly with the concentration of the surrounding solution and to be independent of temperature. This contrasts with the behaviour of 5-hydroxytryptamine, the uptake of which is strongly influenced by temperature. The slope of the line is 2.6, and this gives the ratio of the concentration of tryptamine within and without the platelets.

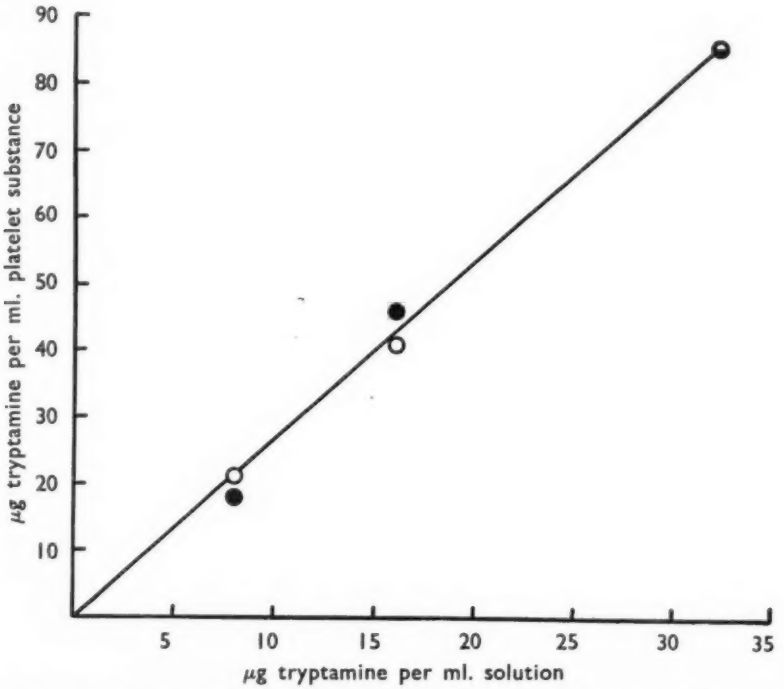

Fig. 2. Uptake of ^{14}C-labelled tryptamine by platelets in 30 min at 0°: ●; at 37° C: ○. Ordinate: μg tryptamine/ml. platelet substance. Abscissa: μg tryptamine/ml. incubation mixture.

The effect of *p*H on the uptake of tryptamine was determined by adding ^{14}C-labelled tryptamine to platelet-rich plasma which had been gassed with oxygen containing 0, 5 and 30% carbon dioxide. After 30 min the *p*H of the plasma and the radioactivity of the platelets were measured. The results are given in Table 4 and show that uptake varied directly with *p*H.

TABLE 4

UPTAKE OF 5-HYDROXYTRYPTAMINE AND TRYPTAMINE BY PLATELETS FROM SOLUTIONS OF THE AMINES IN KREBS SOLUTION

Results are expressed as a percentage of the uptake at *p*H 7·4. Ionization of the amino group was calculated from the data of Vane (1959); pK_a of 5-hydroxytryptamine 10·2, of tryptamine 10·0

	5-Hydroxytryptamine		Tryptamine	
*p*H	% uptake	% unionized	% uptake	% unionized
6·8	130	0·06	64	0·04
7·4	100	0·25	100	0·14
8·0	68	0·99	120	0·63

The effect of tryptamine on the uptake of 5-hydroxytryptamine by platelets. Platelet-rich plasma was gassed with 5% carbon dioxide in oxygen and incubated with each of six different concentrations of tryptamine. A control tube containing 0.9% sodium chloride solution was treated similarly. After 10 min a solution of ^{14}C-labelled 5-hydroxytryptamine in 0.9% sodium chloride solution was added to each tube to give a 10^{-5} M concentration and incubation continued for a further 20 min. In the tubes containing tryptamine the platelets were found to have taken up less 5-hydroxytryptamine than in the tubes containing no tryptamine. In Fig. 3 the uptake is plotted against the molar concentration of tryptamine in the tube. Over a large part of the range the uptake was proportional to the log. of the concentration of tryptamine. From this and from similar experiments it was found that

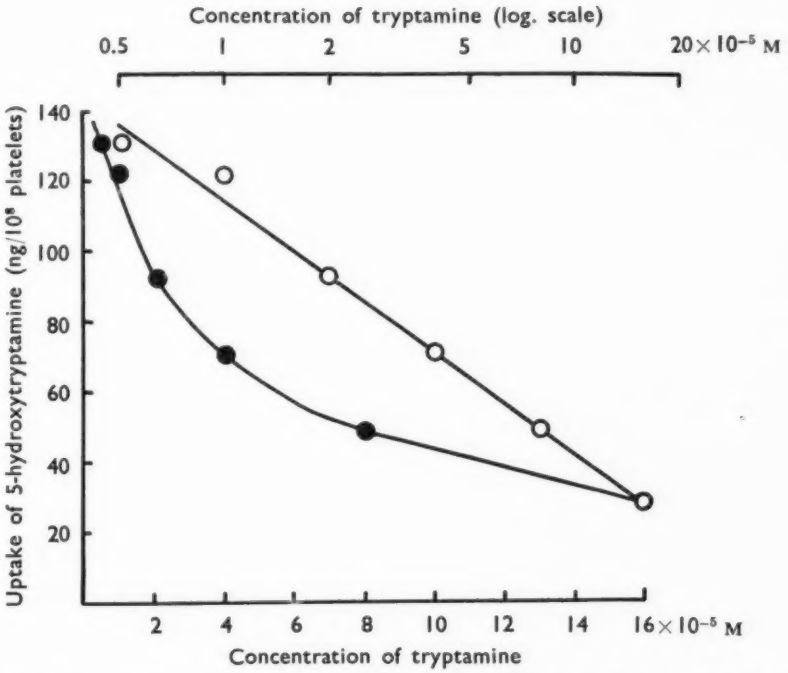

Fig. 3. Effect of various concentrations of tryptamine on the uptake of ^{14}C-labelled 5-hydroxytryptamine by platelets from a 10^{-5} M solution in 20 min. Arithmetical scale ●——●; log scale ○——○. Ordinate: Uptake of 5-hydroxytryptamine ng/10^8 platelets. Abscissa: Concentration of tryptamine.

under the conditions described 50% inhibition of uptake was caused by a concentration of 4×10^{-5} M tryptamine.

In other experiments the concentration of 5-hydroxytryptamine was varied in a series of tubes and the concentration of tryptamine kept the same. The resulting uptake was compared with that in a second series of tubes in which the concentration of 5-hydroxytryptamine was similarly varied but 0.9% sodium chloride solution

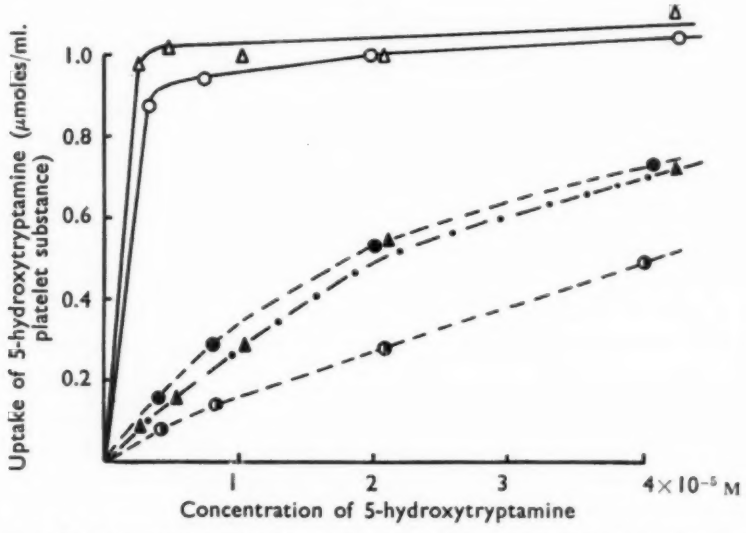

Fig. 4. Uptake of 5-hydroxytryptamine by platelets in 20 min at various concentrations of ^{14}C-labelled 5-hydroxytryptamine in the absence of an inhibitor (continuous lines) and in the presence of tryptamine (5×10^{-5} M) ▲– . –▲ ; cocaine ($2{\cdot}4 \times 10^{-5}$ M) ●– – –● and imipramine ($1{\cdot}9 \times 10^{-6}$ M) ◑– – –◑. Expt. 1: triangles; expt. 2: circles. Ordinate: Uptake of 5-hydroxytryptamine μmoles/ml. platelet substance. Abscissa: Concentration of 5-hydroxytryptamine in the incubation mixture.

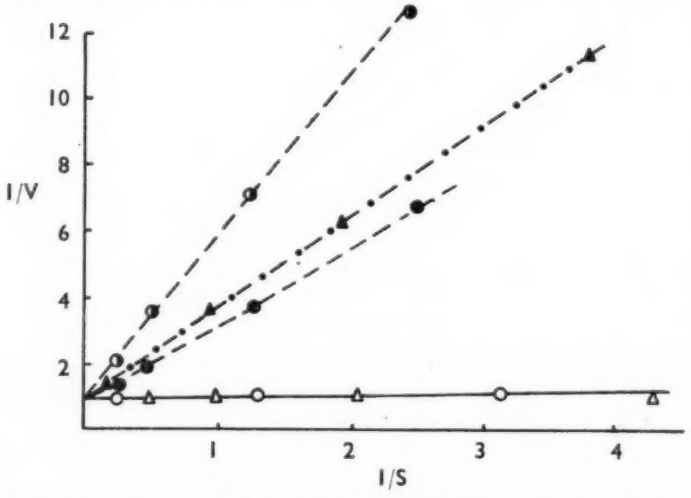

Fig. 5. Ordinate: the reciprocal of the uptake of 5-hydroxytryptamine during the first 20 min incubation (1/V). Abscissa: the reciprocal of the concentration of 5-hydroxytryptamine (1/S) in the absence of an inhibitor and in the presence of tryptamine, cocaine, and imipramine. Concentrations and symbols as in Fig. 4.

was added instead of tryptamine. The results of such an experiment are shown in Fig. 4. In the tubes which contained no tryptamine, uptake changed little over the range 0.25×10^{-5} M to 10^{-4} M 5-hydroxytryptamine, whereas in the presence of 5×10^{-5} M tryptamine it increased considerably with increasing concentration of 5-hydroxytryptamine. If the uptake during the first 20 min is taken as the initial velocity of uptake and the data treated by the method of Lineweaver & Burk (1934) by plotting the reciprocal of the velocity (1/V) against the reciprocal of the concentration of 5-hydroxytryptamine (1/S), Fig. 5 is obtained. From this it is found that both regression lines have the same ordinate intercept; that is, the uptake at infinite 5-hydroxytryptamine concentration is the same whether tryptamine is present or not. This is in agreement with inhibition being competitive.

The effect of various other substances on the uptake of 5-hydroxytryptamine by platelets. The substances listed in Table 5 were tested for inhibitory action by the method described for tryptamine, and the concentration giving 50% inhibition

TABLE 5

CONCENTRATION CAUSING 50% INHIBITION OF 5-HYDROXYTRYPTAMINE UPTAKE BY PLATELETS

Uptake was measured after 20 min incubation at 37° C with 0·7 to $0{\cdot}9 \times 10^{-5}$ M 5-hydroxytryptamine. The following compounds produced inappreciable inhibition at 5×10^{-4} M: histamine, hyoscine, morphine, L-tryptophan, D,L-5-hydroxytryptophan, D,L-dihydroxyphenylalanine, iproniazid, chlordiazepoxide, ouabain

	$\times 10^{-5}$ M		$\times 10^{-5}$ M
Imipramine	0·05	Lysergic acid diethylamide	13
Cocaine	2·5	2-Bromolysergic acid diethylamide	13
Chlorpromazine	3·5	Dopamine	15
Tryptamine	4·0	Methysergide	15
Chlorprothixene	5·0	Atropine	40
Dihydroergotamine	7·5	Trifluoperazine	50
Benzyloxygramine	9·0	Mescaline	50
Promethazine	11·0		
D,L-Amphetamine	11·0		
Tyramine	11·0		

determined graphically from curves such as those in Fig. 3. The final concentration of 5-hydroxytryptamine in the platelet-rich plasma at the end of incubation was not exactly the same in all experiments, because the amount removed by the platelets varied and could not be foretold. It varied from 0.7 to 0.9×10^{-5} M. In the absence of an inhibitor, uptake over this range is unaffected by concentration, but in the presence of an inhibitor the concentration of 5-hydroxytryptamine influences uptake (Fig. 5). The error introduced is, however, small, and the values given in Table 1 indicate the approximate relative potency of the inhibitors.

The uptake at various concentrations of 5-hydroxytryptamine in the presence of 2.4×10^{-5} M cocaine and 1.9×10^{-6} M imipramine is shown in Fig. 4, and the relationship between the reciprocal of the uptake during the first 20 min to the reciprocal of the concentration in Fig. 5. These results are similar to those with tryptamine.

The inhibitory effect of tryptamine and cocaine was also determined in experiments in which the gain in 5-hydroxytryptamine by platelets was measured fluorimetrically. The results of an experiment in which both methods were used

TABLE 6

UPTAKE OF 5-HYDROXYTRYPTAMINE BY PLATELETS AFTER INCUBATION FOR 20 MIN WITH ^{14}C-LABELLED 5-HYDROXYTRYPTAMINE (10^{-5} M) ALONE AND IN THE PRESENCE OF TRYPTAMINE AND COCAINE

	Uptake in ng by 10^8 platelets incubated with 5-hydroxytryptamine		
		In the presence of	
Method of estimation	Alone	Tryptamine 4×10^{-5} M	Cocaine $2{\cdot}5\times10^{-5}$ M
Fluorimetry	194	99	70
Radioactivity	187	97	75

are given in Table 6. The results with the two methods are the same; hence tryptamine and cocaine in the conditions of these experiments do not cause an appreciable efflux of 5-hydroxytryptamine from platelets.

DISCUSSION

The uptake of 5-hydroxytryptamine by platelets varies inversely with the *p*H of the solution, whether the *p*H is changed by variation in the amount of carbon dioxide in solution or by changing the reaction of phosphate buffer. The decrease in uptake at alkaline *p*H values might be due to a change in the amount of free 5-hydroxytryptamine in solution or to a change in its ionization. The proportion of diffusible 5-hydroxytryptamine has been found to be unaffected by change in *p*H over the range 6.6 to 8.3. Changes in ionization can be calculated from the data provided by Vane (1959). The ionization of the amino group is nearly complete at *p*H 8 and increases by $<1\%$ when the *p*H is reduced to 6.8. The hydroxyl group is 99.9% unionized at *p*H 8, and this is increased by $<0.1\%$ when the *p*H is changed to 6.8. In neither case does change in ionization seem adequate to explain the change in uptake. It seems therefore likely that the platelets themselves are sensitive to change of *p*H.

The radioactivity found in platelets after they had been incubated with the ^{14}C-labelled compound was found to be equivalent to their increased 5-hydroxytryptamine content. Since they are unable to synthesize 5-hydroxytryptamine, this indicates that there was no appreciable loss, during the period of incubation, of the 5-hydroxytryptamine originally contained in the platelets, nor was there any loss when tryptamine and cocaine were also present. The possibility of an escape of some of the 5-hydroxytryptamine absorbed during incubation is not excluded.

The uptake of 5-hydroxytryptamine has two striking features: it takes place rapidly even at very low plasma concentrations and continues against a concentration gradient of several hundred to one; secondly, it is very sensitive to temperature change (Hardisty & Stacey, 1955). The optimum temperature for this process was found to be 40° C and the temperature coefficient to be about 3. This suggests that uptake is dependent on the metabolism of platelets, probably for the supply of energy for transport against the concentration gradient. This is supported by the finding that uptake is inhibited by various metabolic inhibitors (Born & Gillson, 1959; Weissbach & Redfield, 1960). In anaerobic conditions uptake continues

unaffected. This is to be expected from the investigations of Waller, Löhr, Grignani & Gross (1959) on platelet metabolism. They found that glycolysis preponderates over the oxidative metabolism of glucose and that only 1/5 of the metabolized glucose could be accounted for by the production of respiratory carbon dioxide. The observation that uptake was unaffected by a 10-fold dilution of plasma with glucose-free Krebs solution does not conflict with the view that the requisite energy comes from glycolysis because the remaining glucose might well have been adequate to maintain metabolism for 20 min incubation. According to Waller *et al.* (1959), the glucose consumption of human platelets is only 75×10^{-6} moles/10^{11} platelets/hr. However, platelets also contain a large amount of adenosine triphosphate which may act as the immediate source of energy.

The evidence that 5-hydroxytryptamine is associated in platelets with adenosine triphosphate which provides the negative charges of a cation exchange system has been presented by Born & Gillson (1959). This has been further strengthened by the presence of 5-hydroxytryptamine and adenosine triphosphate in the same fraction when platelet homogenates are centrifuged through a sucrose density gradient (Baker, Blaschko & Born, 1959). That the combination of these two substances is the sole reason for the concentration of 5-hydroxytryptamine in platelets is made unlikely by the high temperature coefficient, and an active transport system at the platelet surface, as suggested by Hughes & Brodie (1959), is probable. There are thus three stages at which the uptake of 5-hydroxytryptamine might be blocked: (*a*) by inhibition of platelet metabolism, (*b*) at the transport mechanism at the platelet surface, (*c*) at the storage sites.

The uptake of tryptamine by platelets is very different from that of 5-hydroxytryptamine: the concentration gradient is low and does not vary with the concentration of tryptamine, and there is no difference in the rate of uptake at 0° and 37° C. These features suggest a physical process not dependent on platelet metabolism. Moreover, uptake *increases* with rising *p*H, and it is of interest that there is 15 times as much of the unionized form at *p*H 8 as at *p*H 6.8.

Low concentrations of tryptamine depress the uptake of 5-hydroxytryptamine by platelets, and it is now necessary to consider at which of the three sites mentioned above it acts. A consideration of the quantities of the amines involved throws some light on this. Platelets are very nearly saturated with 5-hydroxytryptamine after 90 min incubation, and Pare, Sandler & Stacey (1960) found that human platelets after this period contained 1.3 μmoles/ml. platelet substance. This gives an estimate of the total storage capacity of platelets. In experiments described above it was found that tryptamine reaches a concentration in platelet substance 2.6 times that in the surrounding fluid. When the latter is 4×10^{-5} M, the concentration of tryptamine in platelet substance would be 0.10 μmoles/ml. If all this tryptamine were used in blocking storage sites, only 1/12 of the sites would be blocked. Nevertheless, this concentration of tryptamine causes a 50% inhibition of 5-hydroxytryptamine uptake. From this calculation it appears unlikely that the inhibitory action of tryptamine is exerted on the storage mechanism. Nor is it likely that tryptamine will depress glycolysis or any other energy-producing mechanism. It therefore appears most likely that the inhibitory action of tryptamine

is exerted by blocking the transport sites. This is supported by the kinetics of the reaction, which give a classical picture of competitive inhibition when the results are plotted according to Lineweaver & Burk (1934); however, this may be a deceptive simplification of the overall process.

Of the other substances found to have an inhibitory action, some have structural similarities to tryptamine (tyramine and dopamine) and others antagonize the actions of 5-hydroxytryptamine by competitive inhibition elsewhere in the body (dihydroergotamine, lysergic acid diethylamide, 2-bromolysergic acid diethylamide and benzyloxygramine). Recently evidence has been produced by Trendelenburg (1959), Muscholl (1960) and Whitby, Hertting & Axelrod (1960) that cocaine depresses the uptake of noradrenaline, injected intravenously, in cats and rats. The inhibition of 5-hydroxytryptamine uptake by platelets might thus be another manifestation of this action. Imipramine was by far the most potent of all the substances investigated, and its action has already been reported by Marshall, Stirling, Tait & Todrick (1960). Structurally it is close to the phenothiazines, of which chlorpromazine was the most effective, and to chlorprothixene. All three substances have a dimethylpropylamine chain attached to a condensed three-ring system and all have effects on the central nervous system. However, a number of other compounds need testing before the relationship of action to structure can be profitably discussed. It is of interest that the amino-acids, tryptophan, 5-hydroxytryptophan and dopa, were without action.

I am indebted to Dr. M. B. Thorn for helpful discussion; to Dr. Herbert Weissbach, who kindly gave me the ^{14}C-labelled tryptamine used in these experiments; to the Geigy Co. for imipramine; to Roche Products for 5-hydroxytryptamine creatinine sulphate, chlordiazepoxide and chlorprothixene; to Sandoz Products for the lysergic acid derivatives; and to Smith, Kline & French Laboratories for trifluoperazine.

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TACHYPHYLAXIS TO THE ANTI-TETANUS ACTIVITY OF SOME PHENOTHIAZINE COMPOUNDS

BY

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Although chlorpromazine and acepromazine were the most potent suppressants of local tetanus induced in rabbits by intramuscular injection of the toxin of *Cl. tetani*, repeated injections lost their effect (tachyphylaxis) and sometimes even increased muscle activity, as did single large injections of either of the two drugs. As neither tachyphylaxis nor stimulation occurred in spinal animals, these effects might arise from an action in the brain-stem reticular formation on which chlorpromazine has been shown to have both inhibitory and excitatory effects. The possible cause of the tachyphylaxis is discussed. Evidence is advanced to support the view that tachyphylaxis can occur when chlorpromazine is used in the treatment of tetanus in man. Methotrimeprazine was more potent than chlorpromazine, but less prone to induce tachyphylaxis. These qualities rendered it more desirable for clinical use than the other two compounds.

The phenothiazine derivatives are known to abolish muscle spasm of tetanus both in animals (Laurence & Webster, 1958b) and in man (Adams, Wright, Berman & Laurence, 1959). Quantitative assessments of the activity of a number of these compounds in animals have shown acepromazine and chlorpromazine to be the most potent, acepromazine being ten times as active as chlorpromazine.

When repeated doses of both drugs were given to rabbits with local tetanus over periods of up to eight hours, it was found that a dose, initially effective, might become ineffective or even increase muscle activity. This reduced response to repeated doses, or tachyphylaxis, is the principal subject of this report.

METHODS

Local tetanus was induced in one hind limb of rabbits by the injection of 1,250 mouse minimum lethal doses (M.L.D.) of tetanus toxin into the gastrocnemius muscle.

The intensity of muscle spasm was measured by integrated electromyography which allows the level of tetanus to be expressed in units of activity per minute (Laurence & Webster, 1958a).

Tetanus was maintained at a constant level by employing either an auditory and vibratory stimulus (Laurence & Webster, 1958a), or, in the case of spinal animals, an electrical stimulus. Body temperature of the animals was maintained by electrical radiant heaters throughout the experiment.

Drugs were given intravenously. In the cross-over method of testing at least 24 hr elapsed between each experiment on the same animal. Counts of activity were made during ten

consecutive control periods each of 1 min before each experiment, and then minute counts of muscle activity were recorded for 20 min after the first injection. Subsequently, in prolonged experiments, aggregate counts for 10 min periods were alternated with minute counts for 5 min.

RESULTS

Tachyphylaxis to, and stimulation by, chlorpromazine and acepromazine. An attempt was made to suppress tetanus almost continuously for approximately 8 hr with repeated doses of 1 mg/kg of either acepromazine or chlorpromazine. Injections were made whenever activity returned to 75% of the untreated control value. It was not always easy to apply this criterion, for activity often returned to, and remained for some while at, just below 75% of the control value. On these occasions a further injection was made if activity remained at the same level for 30 min.

On other occasions, when an injection was followed by increased muscle activity, as sometimes happened, another injection was not made immediately but only after the stimulation had lasted for a few minutes and was unequivocal.

The effect of repeated doses of chlorpromazine on the integrated electromyogram is shown in Fig. 1. It can be seen that activity increased above the control after the third, continued to increase after the fourth, and was barely affected by the fifth dose. The result of a similar experiment with acepromazine is shown in Fig. 2. A relatively high dose was used which soon ceased to have any material suppressant effect.

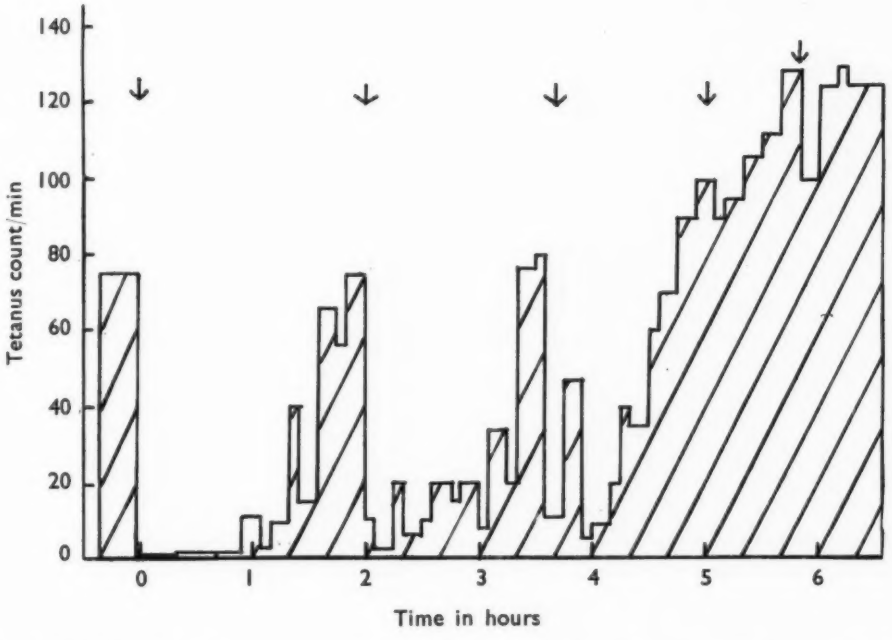

Fig. 1. Tachyphylaxis to chlorpromazine in experimental tetanus. The time course of tetanus activity in a rabbit receiving 1 mg/kg injections of chlorpromazine at the arrows is shown. A distinct tachyphylaxis developed.

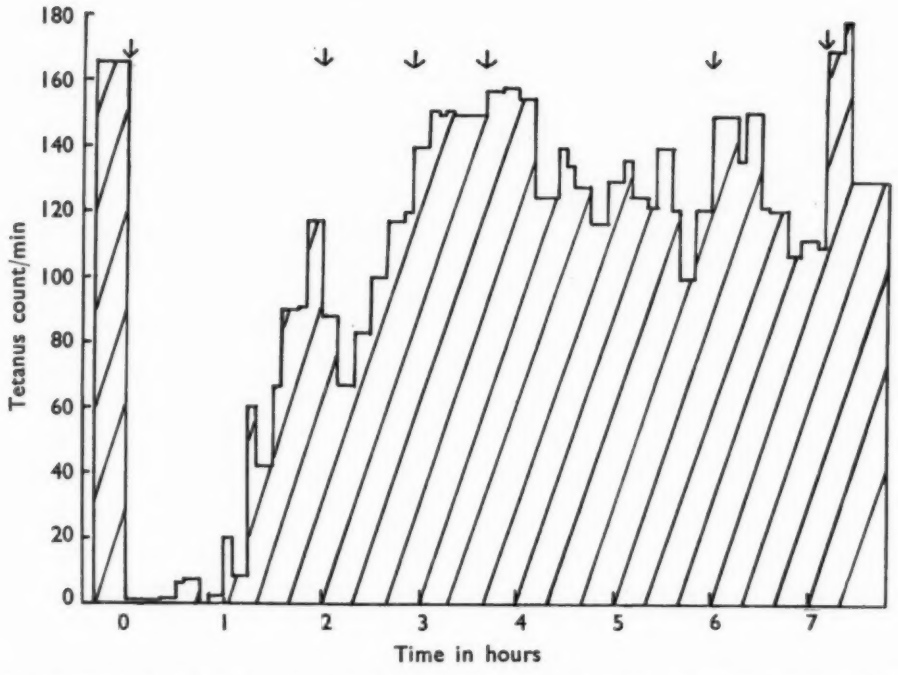

Fig. 2. Tachyphylaxis to acepromazine in experimental tetanus. The time course of tetanus activity in a rabbit receiving 1 mg/kg injections of acepromazine at the arrows is shown. A marked tachyphylaxis developed.

In all except one experiment (BE 59), chlorpromazine (n = 6) (1 mg/kg) was followed by a decrease in activity, however slight or transient (Table 1). On the other hand, acepromazine in the same dose invariably caused immediate stimulation in the third or subsequent injection. In 2 further experiments a smaller dose of acepromazine (0.2 mg/kg) also showed tachyphylaxis even though the total dose did not exceed 1 mg/kg, a dose which, when given initially, abolished tetanus activity almost completely.

TABLE 1

REDUCTIONS IN TETANUS ACTIVITY FOLLOWING SUCCESSIVE DOSES OF ACEPROMAZINE AND CHLORPROMAZINE

Reductions in activity for the 1st hour after each injection were calculated in comparison with the level of activity immediately before *that* injection. Negative values indicate stimulation

		Percentage reduction								
		Chlorpromazine injection					Acepromazine injection			
Animal	Dose (mg/kg)	1	2	3	4	Dose (mg/kg)	1	2	3	4
B58	1·0	93	58	93	43	1·0	92	31	−26	—
B59	1·0	97	84	71	−11	1·0	98	21	−28	4
B64	1·0	82	61	39	68	1·0	85	−40	−19	15
B65	1·0	96	92	68	54	1·0	87	−3	−24	11
						Mean	90	2	−24	10
B82	1·0	91	61	—	—	0·2	70	25	17	28
B83	1·0	87	68	66	81	0·2	54	18	6	8
Mean		90	66	63	43	Mean	62	21	11	18

TABLE 2

REDUCTIONS IN TETANUS ACTIVITY FOLLOWING SUCCESSIVE DOSES OF ACEPROMAZINE AND CHLORPROMAZINE

Reductions in activity for the 1st hour after each injection were calculated in comparison with the level of activity immediately before the 1st injection. Negative values indicate stimulation

		Percentage reduction								
		Chlorpromazine injection					Acepromazine injection			
Animal	Dose (mg/kg)	1	2	3	4	Dose (mg/kg)	1	2	3	4
B58	1·0	93	67	93	55	1·0	92	44	25	—
B59	1·0	97	83	65	−40	1·0	98	45	9	13
B64	1·0	82	75	57	80	1·0	85	8	25	52
B65	1·0	96	95	82	79	1·0	87	31	22	18
						Mean	90	32	20	28
B82	1·0	91	61	—	—	0·2	70	52	50	46
B83	1·0	83	68	66	81	0·2	54	50	37	36
Mean		90	75	73	51	Mean	62	51	43	45

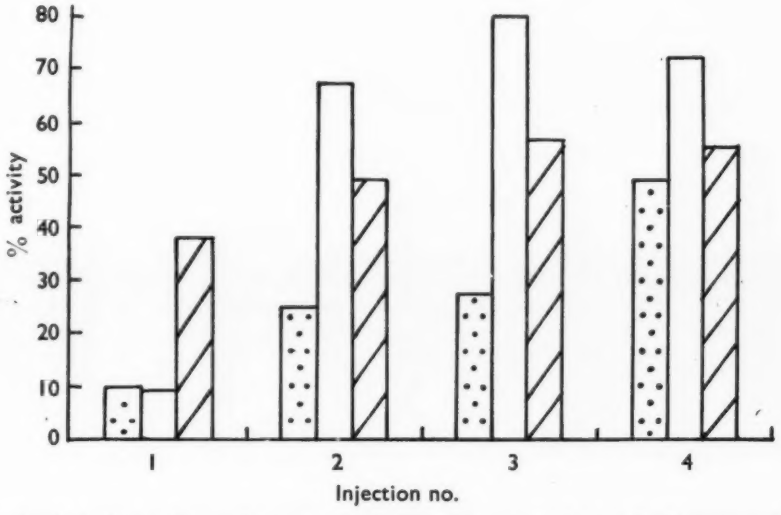

Fig. 3. Tachyphylaxis to the anti-tetanus activity of acepromazine and chlorpromazine. Mean results are plotted for 1 mg/kg chlorpromazine (dotted columns) (6 animals), 1 mg/kg acepromazine (white columns) (4 animals) and 0.2 mg/kg acepromazine (hatched columns) (2 animals). The interval between injections varied from animal to animal, being between 1·5 and 2 hr. Percentage activity values were calculated for the 1st hour after each injection compared with the level of activity before the 1st injection. Although tachyphylaxis developed with all doses, activity did not exceed the control level.

From Table 2 and Fig. 3, it can be seen that tachyphylaxis developed more rapidly with acepromazine than it did with chlorpromazine.

The immediate stimulation which often followed acepromazine injections is shown when the results in Table 1 are plotted (Fig. 4).

Once the tetanus-suppressing activity of either drug began to diminish, increasing the dose by up to five times not only failed to reduce activity but usually stimulated it.

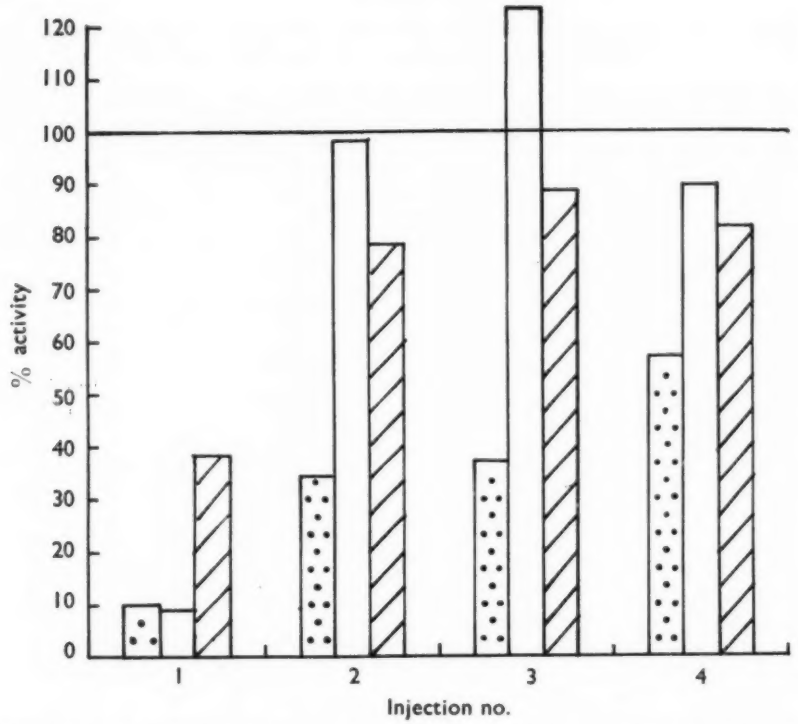

Fig. 4. Tachyphylaxis to the anti-tetanus activity of acepromazine and chlorpromazine. Mean results are plotted for 1 mg/kg chlorpromazine (dotted columns) (6 animals), 1 mg/kg acepromazine (white columns) (4 animals) and 0·2 mg/kg acepromazine (hatched columns) (2 animals). The interval between injections varied from animal to animal, being between 1·5 and 2 hr. Percentage activity values were calculated for the 1st hour after each injection, compared with level of activity before *each* injection. Considerable stimulation occurred after the 3rd 1 mg/kg dose of acepromazine, but activity did not exceed the control level.

Control injections of saline did not produce any consistent change in activity in two animals.

Cross-tachyphylaxis. In 3 experiments in which tachyphylaxis had developed to chlorpromazine, acepromazine failed to reduce tetanus (Fig. 5) and vice versa.

Effect of other drugs after tachyphylaxis had developed. When tachyphylaxis to these phenothiazines had developed, both thiopentone (Fig. 5) and mephenesin retained their usual suppressant effect on tetanus.

Optimal anti-tetanus dose of chlorpromazine and acepromazine. The experiments demonstrating tachyphylaxis suggest that there may be an optimum dose of chlorpromazine and of acepromazine for controlling tetanus, and that to exceed this may not merely offer no advantage but may even be positively disadvantageous.

Three doses of chlorpromazine (1.0, 5.0, 10.0 mg/kg) and of acepromazine (1.0, 2.5, 5.0 mg/kg) were therefore given singly to 3 animals with local tetanus, and the percentage reduction of muscle activity calculated for both the first and second hours after each injection. From the results shown in Fig. 6, it appears that there

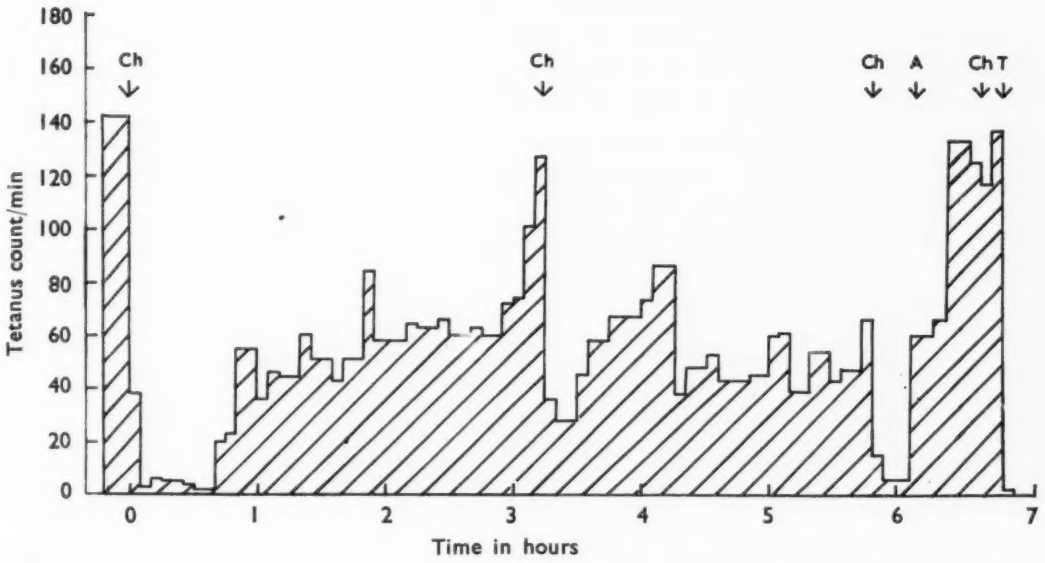

Fig. 5. Cross-tachyphylaxis to the anti-tetanus activity of acepromazine and chlorpromazine. The effect of acepromazine 1 mg/kg (A) and chlorpromazine 1 mg/kg (Ch) on the time-course of tetanus activity is shown. Acepromazine stimulation after chlorpromazine and the normal effect of thiopentone 6 mg/kg (T) are shown.

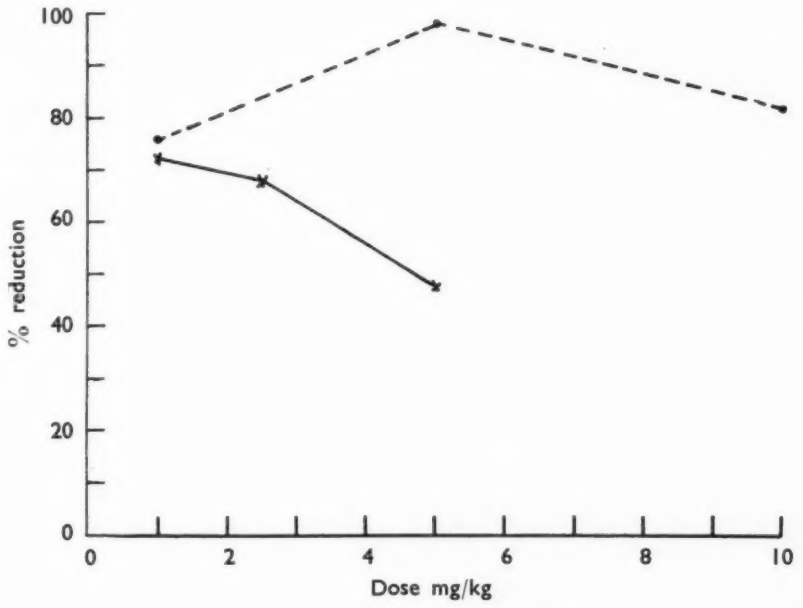

Fig. 6. Optimal doses for the anti-tetanus activity of acepromazine (×——×) and chlorpromazine (●– –●). Percentage reductions were calculated for the 2 hr period after each injection. Each point is the mean of results obtained from at least 3 animals. A sharp decline in the activity of acepromazine above 1 mg/kg is shown.

seems to be an optimal dose for each compound in rabbits, 5 mg/kg for chlorpromazine and at, or just below, 1 mg/kg for acepromazine. Above 1 mg/kg chlorpromazine is more effective than acepromazine, whereas at lower doses the converse is true (Laurence & Webster, 1958b).

If the results of the above experiment are combined with those obtained in the assay of the anti-tetanus activity of these two drugs by Laurence & Webster (1958b), a full dose-response relationship can be plotted (Fig. 7). The slope for chlorpromazine is much steeper than that for acepromazine.

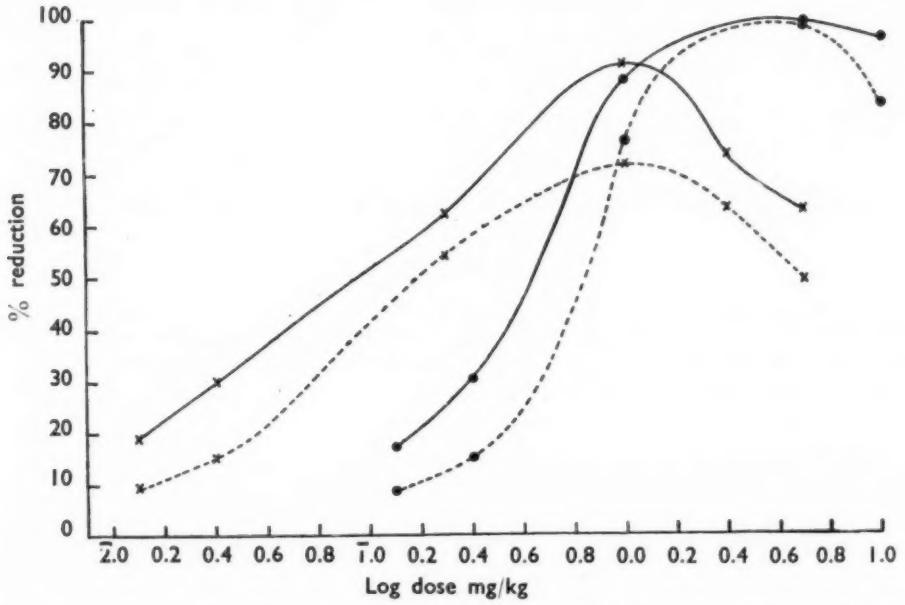

Fig. 7. Dose-response relationship for the anti-tetanus activity of acepromazine and chlorpromazine. Each point is the mean of at least 3 observations. Percentage reductions were calculated for the 1st (acepromazine ×——×; chlorpromazine ●——●) and 2nd hr (acepromazine × - - - ×; chlorpromazine ● - - - ●) after injection. Values for the two lower doses were estimated from data obtained in previous experiments.

The method of plotting results, used above, does not show whether the reduced effectiveness of large doses of acepromazine was due to a reduction in depth of suppression or in duration of action. From the plot of the time-courses of muscle activity following 1.0, 2.5 and 5.0 mg/kg acepromazine (Fig. 8), it can be seen that, although the initial depression of activity was similar with all three doses, the higher doses provided slightly less depression than did the optimal dose of 1 mg/kg. The duration of action, however, decreased with higher doses. The secondary depression of activity at 1.75 hr after 2.5 mg/kg of acepromazine was common to all three animals.

An experiment related to the possible clinical importance of tachyphylaxis. Since chlorpromazine has been advocated in the routine control of clinical tetanus (Laurence, Berman, Scragg & Adams, 1958; Adams *et al.*, 1959; Packard, Cartmill & Henry, 1958), it would be useful to know whether tachyphylaxis occurs in man.

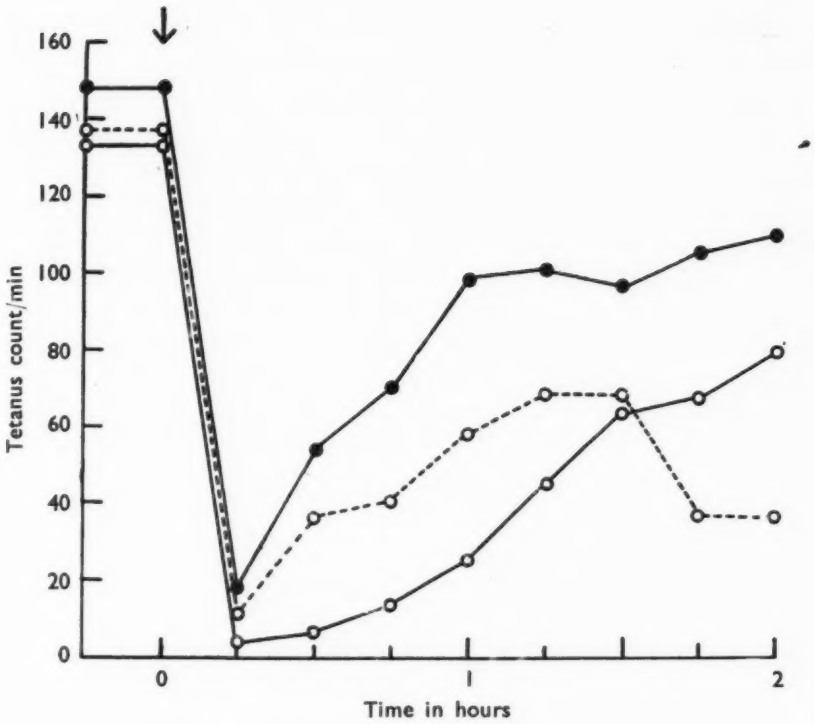

Fig. 8. Mean time-course of the anti-tetanus activity of varying doses of acepromazine given at the arrow (●——● 5 mg/kg; O - - - O 2·5 mg/kg; O——O 1 mg/kg). Mean results for 3 animals are plotted. Although a similar initial depression was obtained with each dose the subsequent rise in activity increased in proportion to dose.

It was recalled by one of us that chlorpromazine, even in large and frequently repeated doses, had notably failed to control very severe tetanus in one patient treated under the care of Professor E. B. Adams during a therapeutic comparison of chlorpromazine with barbiturates (Laurence *et al.*, 1958). This man received, over 5 hr, a total of approximately 16 mg/kg of chlorpromazine intravenously in 6 doses. His convulsions, inadequately controlled from the start, became worse and he died.

Chlorpromazine, on a similar mg/kg basis, was given to each of two rabbits with local tetanus at the same time intervals as the patient received his injections. Tachyphylaxis occurred in both experiments and chlorpromazine ceased to have any useful suppressant effect. The time-course of tetanus activity in one experiment is shown in Fig. 9. It is clear that doses of chlorpromazine, which have been used in man, are capable of inducing tachyphylaxis (and possibly stimulation) in experimental tetanus.

Qualitative differences between experimental tetanus and tetanus activated by phenothiazines. When tetanus was activated by a phenothiazine derivative, muscle activity differed from normal and often bore no relationship to the standard afferent stimulus used to activate it. Spontaneous tremors and twitches sometimes occurred

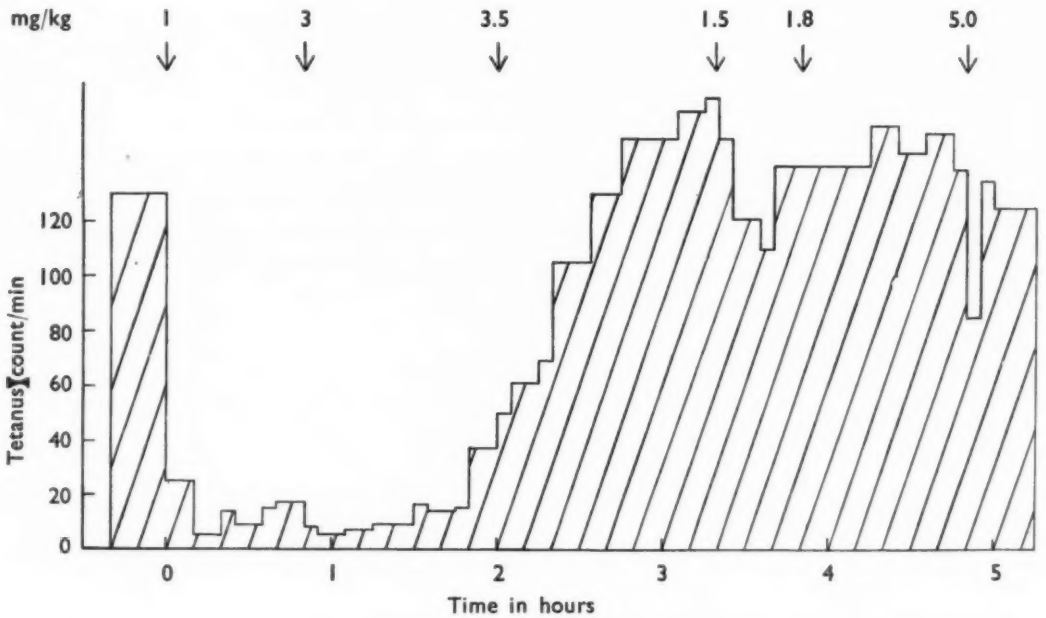

Fig. 9. Time-course of an experimental demonstration of the possible clinical importance of tachyphylaxis. Doses of chlorpromazine given to a patient with general tetanus expressed in mg/kg and injected into a rabbit at the arrows, with local tetanus at the same time-intervals as they were received by the patient, in whom they became ineffective. Stimulation occurred after the 3rd injection.

in both the tetanus and control limbs, and occasionally muscle activity in the control limb even exceeded that in the affected one.

As the depressant effect of a large dose (for example, 5 mg/kg or more) wore off, it was usually followed by an increase in activity above the control level and, if a second dose was given during the subsequent 24 hr, this was often followed by immediate activation. This activated tetanus could be abolished by mephenesin or a barbiturate drug.

In normal rabbits receiving large doses of acepromazine (5 mg/kg) or chlorpromazine (10 mg/kg), tremors and twitches sometimes occurred, but were only rarely associated with sustained regular hyperactivity.

Tachyphylaxis in the spinal rabbit. In order to discover whether tachyphylaxis could be demonstrated in the spinal animal with acepromazine, four rabbits underwent spinal section in the lower thoracic region under thiopentone and ether anaesthesia and tetanus toxin was injected intramuscularly 48 hr later. Following operation they required careful nursing with expression of urine from the bladder at least once daily. On recovery, the level of tetanus activity in the spinal animal was always substantially below that in the intact animal, and it was necessary to use an electrical cutaneous stimulus to maintain regular activity. Electrode polarity was regularly reversed to avoid alteration in the stimulus due to polarization of the electrodes.

When acepromazine was given, neither tachyphylaxis nor stimulation was now observed in any of the 4 animals, but tetanus was reduced by about 60%.

Other phenothiazines. As tachyphylaxis and stimulation are clearly undesirable properties of an anticonvulsant, a number of other phenothiazines, including perphenazine, trimeprazine, and methotrimeprazine (Veractil), were tested for potency (unpublished observations). Promethazine and promazine had been previously compared with chlorpromazine (Laurence & Webster, 1958b). The only compound, apart from acepromazine, found to be more potent than chlorpromazine was methotrimeprazine, which was about twice as potent. A single dose of 1 mg/kg abolished tetanus for 1 to 2 hr in two rabbits and, after 4 hr, the activity had returned to only 30% of normal. Further doses showed no or only slight tendency to stimulate activity and tachyphylaxis seemed less. A single injection of 5 mg/kg of methotrimeprazine completely abolished all activity for 4 hr, but tremors followed after 10 mg/kg.

These results suggest that methotrimeprazine may be superior as an anticonvulsant to chlorpromazine for use in clinical tetanus. Opportunity has since arisen to treat a case of clinical tetanus with methotrimeprazine. A dose of 25 mg given intravenously enabled the patient to increase the jaw separation from 1.1 cm to 2.5 cm with symptomatic relief. During succeeding days doses of 25 to 50 mg given intravenously to a daily total of 400 mg appeared to relieve spasms, but no opinion on its clinical merit can be made on the basis of this single case.

DISCUSSION

Tachyphylaxis occurs with certain drugs such as nicotine and ephedrine, although it is doubtful whether there is a common underlying mechanism. The tachyphylaxis to the anti-tetanus activity of repeated doses of 1 mg/kg of acepromazine and of chlorpromazine reported here may be explained on the assumption that these phenothiazine derivatives may both depress and stimulate the central nervous system and that the time courses of these two actions differ considerably. The interrelationship between inhibition and excitation is probably complex, and excitation may only become obvious when the normal balance between inhibition and excitation in the central nervous system has been disturbed, as in tetanus. Although it has been shown that there is an optimal effective dose for both acepromazine and chlorpromazine and that large doses may become ineffective or even stimulate activity, it seems unlikely that tachyphylaxis is simply due to the accumulation of doses to exceed the optimal and produce excitation. Tachyphylaxis still occurred to the repeated injections of small doses of acepromazine, despite the fact that the first injection was only slightly effective and that the total dose did not reach the optimal single depressant dose. Also when 1 mg/kg of acepromazine was injected after four similar doses an immediate stimulation was obtained whereas a single injection of 5 mg/kg produced depression.

This latter observation and the fact that a large dose of acepromazine given 24 hr after a number of smaller doses resulted in immediate stimulation instead of the expected depression suggest that excitation lasts much longer than inhibition. Consequently, even though the potential excitatory action of any one dose is

probably considerably weaker than the inhibitory action, since the phenothiazines are primarily depressants, persistence of excitation after depression has worn off will ensure an accumulated effect with repeated doses which will gradually reduce the depressant effect of succeeding doses and eventually counteract the inhibitory effect of any one dose and produce stimulation. This would explain why tachyphylaxis occurred to the repeated injection of small as well as large doses.

That phenothiazine excitation is a distinct effect and not simply a loss of initial inhibition is also suggested by the finding that the activity recorded once tachyphylaxis has developed differs from typical tetanus. Twitches or tremor, seldom seen in tetanus, were often recorded either alone or superimposed on tonic activity, but the characteristic reflex response to afferent stimulation, obtained in the tetanus animal, was absent. Activity also often developed in a previously quiescent control limb, sometimes exceeding that in the affected limb, and brief stimulation was obtained in normal animals with large doses of both compounds.

The relationship between chlorpromazine-induced tremors and hypothermia has been discussed by Domer & Feldberg (1960). The possibility that hypothermia may have played a part in some of the experiments reported here has not been excluded but is not considered to be important, even if it occurred, since excitation immediately followed some injections and single large doses of phenothiazines had less suppressant activity on tetanus than smaller doses.

Another reversal of central effect with increased doses has been recorded by Rinaldi & Himwich (1955), who found that chlorpromazine depressed the electrical activity of the brain and the arousal response by an action on the reticular system in doses up to 10 mg/kg, but that higher doses had the reverse effect. As neither tachyphylaxis nor stimulation could be obtained in spinal animals in the present experiments, although they probably occur in decerebrate preparations (unpublished observation), phenothiazine stimulation would appear to be limited to the reticular system of the brain-stem. If this is so, tachyphylaxis may be only another example of the numerous extrapyramidal side-effects, similar to parkinsonism, that have been widely reported in patients receiving large doses of chlorpromazine (Moyer, Wright & Finney, 1955; Hall, Jackson & Swain, 1956; Giacobin & Lassenius, 1955; and Goldman, 1955).

Phenothiazine stimulation of the reticular system may involve the adrenergic mechanism which has been shown to exist at this level of the nervous system (Bonvallet, Dell & Hiebel, 1954; Hiebel, Bonvallet & Dell, 1954; Rothballer, 1956; Bradley, 1957), for, as pointed out by Himwich (1958), the structural formulae and in particular the side-chain of chlorpromazine and adrenaline are very similar. This may account for the fact that the phenothiazine derivatives are, to varying extents, antiadrenaline agents, and why acepromazine, which is the more active antiadrenaline derivative (Schmitt, Mercier, Aurousseau, Hallot & Comoy, 1957; Mercier, Schmitt, Navarro, Gavend & Gavend, 1958), also produced the most stimulation. Any relationship between the antiadrenaline activity of the phenothiazines and their effects in tetanus must be speculative. Since the most common examples of tachyphylaxis occur in the use of the sympathomimetic amines, it is of interest that tachyphylaxis has been recorded to the hypotensive antiadrenaline effects of chlor-

promazine both in man and animals (Dundee, 1958 ; Cahn, George & Pierre, 1957 ; Roth, Irwin, Eckhart, Tabacknick & Govier, 1959).

These results show that there is an optimal frequency of administration as well as an optimal dose for continuous suppression of tetanus in rabbits. This may be of considerable clinical importance, for the pharmacology of the phenothiazine compounds in rabbits and in man seems to be similar. That tachyphylaxis and perhaps also activation of tetanus by chlorpromazine might occur in man is strongly suggested by the present work. Barr (1958) and Packard *et al.* (1958) have both suggested that tolerance may occur in man.

Because acepromazine was more active than chlorpromazine, it was given preliminary clinical trial (Adams *et al.*, 1959) before it had been found to have the potential disadvantages reported here. It did not appear to be more than an alternative to chlorpromazine. The present experiments suggest that, had acepromazine been subjected to formal clinical trial, it might have proved unsatisfactory because of the occurrence of tachyphylaxis and stimulation.

The only other phenothiazine derivative found to be more active against experimental tetanus than chlorpromazine was methotrimeprazine. The results reported here indicate that a possible superiority to chlorpromazine in the treatment of clinical tetanus may lie in its greater potency and in its tendency to induce less stimulation. It is of interest to note that this greater tetanus depressant activity is accompanied by an antiadrenaline activity approximately equal to that of chlorpromazine.

Because of the possibility of stimulation by phenothiazine derivatives it seems likely that the combination of a phenothiazine with some other depressant, such as a barbiturate, may be desirable clinically. Such a combination has already been used extensively to control convulsions and found to be more easily handled than chlorpromazine alone, despite the findings by Adams (1958) that it did not reduce mortality materially when the mixture was compared with either drug alone.

One of us (R. A. W.) is now Stothert Research Fellow of the Royal Society.

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THE EFFECT OF MEPHENTERMINE ON ISOLATED DOG HEARTS, NORMAL AND PRETREATED WITH RESERPINE

BY

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The inotropic activity of the non-catechol sympathomimetic amine, mephentermine sulphate, on the failing dog heart-lung preparation, was 1/10 to 1/20 that of adrenaline. Mephentermine showed no inotropic effect on preparations from animals pretreated with reserpine. The chronotropic and "calorigenic" actions of mephentermine were tested on modified heart-lung preparations to permit a more accurate measurement of coronary flow, and were found to be greater than its inotropic effect relative to adrenaline. Furthermore, the action of mephentermine was longer-lasting than that of adrenaline. If adrenaline was infused 15 min after the termination of mephentermine administration and when the action of the latter was still at a maximum, a further increase in heart rate and especially oxygen consumption was observed. In preparations from dogs treated with enough reserpine to deplete the heart of noradrenaline, mephentermine had only slight chronotropic and calorigenic actions. However, further addition of adrenaline after a 15 min pause caused a rise in heart rate, oxygen consumption, and coronary flow which almost duplicated the additive effects of both amines on the preparations not treated with reserpine. It would appear that adrenaline acted on its own and in addition "restored" the action of mephentermine on the reserpinized preparations. The action of adrenaline alone on reserpinized preparations was not increased compared with that on normal preparations. These observations are relevant to a consideration of the mechanism of action of non-catechol sympathomimetic amines on the heart, and are in harmony with the concept that mephentermine, a non-catechol amine, requires the presence of added or stored catechol amines for its action. Reserpine treatment did not alter the mechanical efficiency of the heart despite its depletion of noradrenaline.

Mephentermine is N,α,α-trimethylphenethylamine, and its sulphate has found use as a pressor agent in hypotensive states resulting from myocardial infarction and general or spinal anaesthesia (*New and Non-official Drugs*, 1960). It has also been reported to act on the heart. Goldberg, Cotten, Darby & Howell (1953), using intact open-chest, vagotomized dogs, found that mephentermine was about 1/1,000 as active as adrenaline in its inotropic effect, whereas Kukovetz, Hess, Shanfeld & Haugaard (1959) found that mephentermine had no action on the contractile force of the Langendorff perfused heart. In view of these findings and in the hope of contributing to the mechanism of action of non-catechol sympathomimetic amines, experiments were performed to study the effects of mephentermine on isolated dog hearts under the same conditions previously used for comparative studies of adrenaline and noradrenaline (Fawaz & Tutunji, 1960).

METHODS

The inotropic action of mephentermine was tested on the classical Starling heart-lung preparation. Failure was induced within 15 min by repeated additions of pentobarbitone until the systemic output, originally 800 to 900 ml./min, fell to about half this level with a corresponding rise in right auricular pressure. Mephentermine, 100 μg, or adrenaline in doses of 5 or 10 μg was then injected into the venous inflow tube near the right heart or added to the reservoir. Experiments were also carried out on heart-lung preparations made from dogs pretreated with reserpine to see if depletion of catechol amines by reserpine affected the inotropic action of mephentermine. Reserpine was administered intraperitoneally in two consecutive doses of 0.5 mg/kg at intervals of 24 hr, the second dose being given 24 hr before the experiment.

The chronotropic, calorigenic and coronary-vasodilating actions of either mephentermine or adrenaline alone were studied on heart-lung preparations as described previously (Fawaz & Tutunji, 1960). The "calorigenic" action of a sympathomimetic amine is defined as the increase in oxygen consumption which, in the absence of a change in work performed, cannot be explained solely by a rise in heart rate. In these experiments, reserpine was given intraperitoneally in two or three doses of 0.1 mg/kg each at 48 hr intervals, the last dose being given 24 hr before the experiment. We chose this dose because larger doses made the animals very ill and they abstained from eating. Furthermore, Waud, Kottegoda & Krayer (1958) have shown that one dose of 0.1 mg/kg of reserpine was sufficient to deplete the heart of noradrenaline in 24 hr.

The mechanical efficiency of the heart was calculated on the basis that 1 ml. of oxygen consumed/2 kg-m of work performed constituted 100% efficiency.

Mephentermine sulphate, L-adrenaline bitartrate, and reserpine ascorbate (kindly supplied by Wyeth, Sterling-Winthrop and Ciba respectively) were used in these experiments, but the dosages always refer to the base.

RESULTS

The inotropic actions of mephentermine and adrenaline

The relative inotropic actions of mephentermine and adrenaline were assessed by their effects in increasing the output and in reducing right auricular pressure in the failing heart. In 4 experiments adrenaline was given first, and after its action had subsided mephentermine was given. In another 4 experiments mephentermine was given first, and then adrenaline. On the basis of these observations it was concluded that 100 μg of mephentermine was roughly equivalent in its maximum effect to 5 to 10 μg of adrenaline, although the action of mephentermine was longer-lasting than that of adrenaline. These doses of mephentermine and adrenaline restored the output of the failing heart to 80 to 100% of its original value and reduced the right auricular pressure correspondingly.

The comparison between mephentermine and adrenaline was difficult because a second injection of one amine was rarely as effective as the first injection, and because there was considerable variation between preparations in the ease with which failure was produced. Despite these inherent sources of inaccuracy it is reasonable to assess the inotropic activity of mephentermine as 1/10 to 1/20 of that of adrenaline in the failing dog heart-lung preparation.

In 4 experiments on heart-lung preparations from reserpine-treated dogs mephentermine was almost entirely without an inotropic action.

TABLE 1

EFFECT OF MEPHENTERMINE ON NORMAL AND RESERPINE-TREATED ISOLATED DOG HEARTS

Mephentermine sulphate was added to the reservoir at a rate of 50 μg of base/min for 15 min, followed by a 15 min interval, then a 15 min infusion of 4 μg adrenaline base/min. The numeral (1) indicates at the start of an experiment, (2) after 15 min, (3) after 30 min, (4) after 45 min. All % changes refer to (1) as a standard. The figures in columns 5(a) and 5(b) refer to the effects of adrenaline given at the start of an experiment at the rate of 4 μg adrenaline base/min for 15 min. The figures in 5(a) were reported by Fawaz and Tutunji (1960)

	Normal hearts (8 experiments)					Reserpine-treated hearts (8 experiments)				
	1	2	3	4	5(a)	1	2	3	4	5(b)
Heart rate Mean±s.e.	141 (±5)	250 (±17)			195 (±11)	131 (±6)				
% change in heart rate Mean±s.e.		78 (±7)	68 (±5)	83 (±5)	48 (±5)		18 (±5)	17 (±4)	92 (±8)	68 (±7·2)
Oxygen consumption (ml./100 g/min)	7·1 (±0·35)					6·3 (±0·28)				
Mechanical efficiency Mean±s.e.	10·9 (±0·6)					11·1 (±0·6)				
% change in oxygen consumption Mean±s.e.		74 (±17)	75 (±12)	143 (±18)	54 (±10)		12 (±6)	20 (±6)	140 (±22)	61 (±10·3)
Mean % change in work performed		−5·0	−3·0	+8·0			0	+1·5	+1·0	
Coronary flow (ml./min) Mean±s.e.	59 (±6)					57 (±6)				
% Change in coronary flow Mean±s.e.		93 (±20)	120 (±17)	320 (±32)	113 (±12)		27 (±10)	75 (±25)	322 (±47)	75 (±10·6)

The chronotropic, calorigenic and coronary-dilator actions of mephentermine

Normal animals. Since the inotropic action of mephentermine was found to be 1/20 to 1/10 that of adrenaline, an amount of mephentermine was infused in the course of 15 min which was 12.5 times the quantity of adrenaline used in a previous publication that dealt with the relative potencies of adrenaline and noradrenaline on the isolated heart (Fawaz & Tutunji, 1960). The results obtained in the previous publication may be used for comparison, because in both studies the initial values for heart rate (138 ± 4.8 and 141 ± 5.2), oxygen consumption (6.7 ± 0.38 and 7.1 ± 0.35) and coronary flow (55 ± 6 and 59 ± 5.8) were very similar. Furthermore, the experimental conditions were similar.

It can be seen from Table 1 that the chronotropic action of mephentermine is greater than its inotropic action, if adrenaline is used as a standard. The average maximum heart rate obtained after the first infusion of 60 μg adrenaline was 195, whereas the figure for 750 μg mephentermine was 250. Furthermore, the action of mephentermine lasted much longer than that of adrenaline, for, while the action of adrenaline subsided within 30 min after the end of the infusion, that of mephentermine continued for at least 1 hr, and there was only a slight decrease in heart rate 15 min after the end of the infusion. If at this stage 60 μg adrenaline was infused in the course of 15 min, there was a further, although small, rise in heart rate over and above the maximum rate obtained at the end of the 15 min infusion period with mephentermine.

What has been said of the chronotropic action of mephentermine applies also to its calorigenic action, which is greater than its inotropic action if adrenaline is taken as a standard. Here, however, the addition of 60 μg adrenaline after the 15 min pause following the mephentermine infusion resulted in a significant increase in oxygen consumption in every experiment over and above the maximum value obtained with mephentermine. The relative increase in oxygen consumption after the additional adrenaline infusion was much greater than the corresponding increase in heart rate. However, no special significance should be attached to this latter finding, since with ventricular rates between 250 and 260 the maximum chronotropic effect that can be attained by the use of drugs is reached.

The increased coronary flow after mephentermine is to be expected in view of the increase in oxygen consumption. Again, our experiments do not answer the question as to whether the sympathomimetic amines studied dilate the coronary vessels by a direct action or as a result of increased cardiac oxygen consumption. In the previous experiments (Fawaz & Tutunji, 1960) a $54 \pm 10\%$ increase in oxygen consumption due to adrenaline was accompanied by a $113 \pm 12\%$ increase in coronary flow. In the present experiments, starting with almost identical values for coronary flow and oxygen consumption, mephentermine caused a $74 \pm 17\%$ increase in oxygen consumption with $93 \pm 20.4\%$ increase in coronary flow. It would appear from this that adrenaline had a direct coronary vasodilator action. However, in view of the magnitude of the standard errors, the author is reluctant to draw such a conclusion.

Reserpine-pretreated animals. Mephentermine had only slight chronotropic and calorigenic effects on the reserpine-pretreated animals. However, the administration

of adrenaline after the termination of a mephentermine infusion resulted in an increase in heart rate, oxygen consumption and coronary flow which was almost identical with that obtained when adrenaline was added on top of mephentermine in preparations from animals not pretreated with reserpine. The response of reserpinized hearts to adrenaline alone is shown in column 5(b) of Table 1, where only the chronotropic action was increased as compared with that on normal hearts, but the calorigenic and coronary dilating actions were even less than in normal hearts. The calorigenic and chronotropic actions of adrenaline on reserpinized hearts subsided after 30 min just as in the case of normal hearts. Table 1 also shows that a dose of reserpine which depleted the heart of noradrenaline had no influence on the mechanical efficiency of the isolated heart.

DISCUSSION

The inotropic action of mephentermine on the isolated heart is 1/10 to 1/20 that of adrenaline, and the ratio is even more in favour of mephentermine when the chronotropic and calorigenic actions are considered. Furthermore, the actions of mephentermine are longer-lasting than those of adrenaline. These findings are in marked contrast with those of Goldberg *et al.* (1953), who reported a ratio of 1 : 1,000 for the inotropic action of mephentermine as compared to adrenaline. Goldberg *et al.*, however, did not use failing isolated hearts but intact open-chest dogs, and assessed the inotropic action by measuring the contractile force by means of levers attached to the right ventricle. We have no explanation to offer for this marked difference in response between the intact animal and the isolated heart.

The ineffectiveness of mephentermine when tested on reserpinized hearts is not surprising in view of observations made by various authors (Burn & Rand, 1958) that non-catechol sympathomimetic amines, such as tyramine and phenylethylamine, lose most of their pressor activity in animals pretreated with reserpine. What was not expected in our experiments was the effect of administering adrenaline after the termination of the mephentermine infusion. The result was an additive effect of both amines similar to and almost identical with that obtained on the preparations not treated with reserpine. One may regard this as a " reactivation " by adrenaline of the effects of mephentermine. It does not support the concept that non-catechol sympathomimetic amines, of which mephentermine is a representative, have no action of their own but only act indirectly either by protecting catechol amines from enzymatic inactivation, or by releasing " stored " catechol amines. In a reserpinized heart with little or no catechol amine content, mephentermine was inactive. Addition of adrenaline restored the action of mephentermine and in addition exerted its own independent action. One conclusion that may be drawn from these experiments is that mephentermine requires the presence of added or " stored " catechol amines for its action.

The observation that the mechanical efficiency of a reserpinized heart is not altered is important in connexion with the question as to whether stored cardiac noradrenaline is " available " for metabolic purposes, and with the extent to which adrenergic and cholinergic substances normally influence the mechanical efficiency of the heart.

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SUPERSENSITIVITY OF SALIVARY GLANDS FOLLOWING TREATMENT WITH BRETYLIUM OR GUANETHIDINE

BY

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The sensitivity of the submaxillary glands to noradrenaline was estimated once a week in cats anaesthetized with hexobarbitone. Daily subcutaneous injections of bretylium or guanethidine were found to produce a supersensitivity of the glands, similar to that caused by extirpation of the superior cervical ganglion.

Prolonged treatment with various drugs interfering with the transmission of nerve impulses causes a supersensitivity to chemical stimuli which resembles that brought about by denervation. The salivary glands have proved useful in studies on "pharmacological denervation" since secretion of saliva seems particularly susceptible to blocking agents. So far, drugs acting on the parasympathetic innervation of the glands have been investigated (see Emmelin, 1961a). The salivary gland cells receive, however, sympathetic secretory fibres also, and they become supersensitive to chemical stimuli when the superior cervical ganglion has been removed (Simeone & Maes, 1939; Emmelin & Engström, 1960a and b). In the present experiments attempts were made to find out whether prolonged administration of sympathetic blocking agents can cause a supersensitivity of the submaxillary gland cells to secretory agents.

Treatment with reserpine has been found to sensitize the heart and various smooth muscles to chemical agents (Burn & Rand, 1958a, b, c; 1959; Trendelenburg & Gravenstein, 1958). In our first experiments on salivary glands we also used reserpine; there was some indication that a supersensitivity developed in some of the cats, but in the course of the prolonged treatment the secretory capacity of the glands tended to diminish as the general condition of the experimental animals deteriorated. We therefore used bretylium instead of reserpine, and later guanethidine when this drug became available. These drugs, however, are known to increase the effects of adrenaline and noradrenaline even in acute experiments (Boura & Green, 1959; Maxwell, Mull & Plummer, 1959; Page & Dustan, 1959; Maxwell, Plummer, Schneider, Povalski & Daniel, 1960; Vernikos-Danellis & Zaimis, 1960); we found this to occur in the salivary glands also. The problem was, therefore, to investigate whether, apart from this acute effect, a sensitization similar to that following sympathetic denervation can develop in chronic experiments.

METHODS

The sensitivity of the two submaxillary glands of cats to noradrenaline was tested in each animal once weekly. The cats were anaesthetized with hexobarbitone sodium (20 mg/kg) administered intracardially after induction with ether and the submaxillary ducts cannulated from the mouth as previously described (Emmelin & Muren, 1952). Further doses of hexobarbitone were given when required. Standard doses of noradrenaline (2, 5, 10 and 20 μg/kg) were injected through the needle in the heart into the left ventricle. Noradrenaline was chosen as a test drug since extirpation of the superior cervical ganglion is known regularly to cause a supersensitivity towards this agent (Emmelin & Engström, 1960b). Bretylium tosylate (Darenthin) and guanethidine sulphate (Ismelin) were administered subcutaneously as described below. For comparison the superior cervical ganglion of one side was excised in some experiments.

RESULTS

The results with bretylium and guanethidine were essentially similar and will be treated together.

Table 1 summarizes the results of a series of experiments in which 10 mg/kg of bretylium (cats 1 to 4) or guanethidine (cats 5 to 11) was given subcutaneously for

TABLE 1

EFFECT OF BRETYLIUM, GUANETHIDINE AND GANGLIONECTOMY

Sensitivity of the submaxillary gland to noradrenaline before and after treatment with bretylium (cats 1 to 4) or guanethidine (cats 5 to 11). The effect of extirpation of the superior cervical ganglion is shown in 6 of the cats

Cat	Noradrenaline μg/kg	Total response to noradrenaline (drops of saliva)		
		Before	After treatment	After ganglionectomy
1	20	0·3	3·3	3·3
2	10	0·3	6·7	8·7
3	20	0·3	5·7	7·5
4	20	2·3	7·0	9·3
5	20	0·7	6·7	—
6	20	0·7	8·5	—
7	10	0·3	4·7	—
8	10	0·7	8·7	—
9	10	1·7	6·7	6·5
10	20	2·7	7·7	5·3
11	20	0·3	6·7	—

a period of 1 to 3 weeks ; the last injection of the drug was given about 24 hr before the sensitivity was estimated. The effect of ganglionectomy in some of the animals is also shown. In all the cases the responses to noradrenaline were found to be augmented after the treatment with bretylium or guanethidine. The increase was of the same order of magnitude as that produced by removal of the superior cervical ganglion. In the table the observation made on each cat is exemplified with one of the standard doses of noradrenaline ; the dose chosen was that which caused a small response in the normal gland. When smaller doses of noradrenaline were tested the threshold was found to be similarly lowered by drug treatment and ganglionectomy.

When in some cases the estimation of the sensitivity was carried out on the day after the first injection of bretylium or guanethidine some increase in responsiveness

to noradrenaline could usually be demonstrated. This was probably the sensitizing effect ordinarily seen in the acute experiment. The effect was considerably smaller than that found after treatment with daily injections for one week. The objection could be made that the effect encountered after one week of treatment was still nothing but the acute one, more pronounced after one week than after one day because a large amount of the drug had accumulated in the body during the week; the two drugs are known to be eliminated remarkably slowly (Boura, Green, McCoubrey, Laurence, Moulton & Rosenheim, 1959; Maxwell, Mull & Plummer, 1959; Maxwell, Plummer, Schneider, Povalski & Daniel, 1960; Boura, Copp, Duncombe, Green & McCoubrey, 1960). Against this explanation two types of results may be quoted.

Firstly, in a series of experiments each cat received single injections of different doses of the drugs with intervals of several weeks between the injections so as to leave time enough for the drug to be eliminated; on the day after each injection the sensitivity of the submaxillary glands to noradrenaline was estimated. Single doses of 10, 30 and 50 mg/kg of bretylium or guanethidine were given. In addition, doses of 100 and 150 mg/kg were administered, but they were divided into two injections over two days (50 + 50 and 50 + 100 mg/kg); most cats tolerated even these large doses. The experiments showed that the sensitizing effect, observed one day after an injection, was not much more pronounced with the larger than with the smaller doses of bretylium or guanethidine. Even after 150 mg/kg the increase in responsiveness to noradrenaline was smaller than that obtained when 10 mg/kg was given daily for a week, that is, when altogether 70 mg/kg was injected.

Secondly, the slow elimination of bretylium and guanethidine made the following type of experiment possible. On the first day 50 mg/kg of the drug was injected and on the next day 50 or 100 mg/kg. The sensitivity to noradrenaline was estimated on the third day and then with weekly intervals. Five such experiments are shown in Table 2 (cats 1 to 4 with guanethidine, cat 5 with bretylium). It can

TABLE 2

PROLONGED EFFECTS OF BRETYLIUM AND GUANETHIDINE

Effect on the sensitivity of bretylium 100 mg/kg (cat 5) or guanethidine 150 mg/kg (cats 1 to 4)

		Total response to noradrenaline (drops of saliva)					
			Time after treatment				
Cat	Noradrenaline μg/kg	Before	1 day	1 week	2 weeks	3 weeks	4 weeks
1	10	1·5	3·7	6·7	4·7	3·0	0·5
2	10	0·3	2·3	5·7	2·3	1·3	0·3
3	10	0·3	2·7	6·3	1·3	—	—
4	5	0	0·3	1·3	0·7	0·3	—
5	10	1·5	0·7	5·7	6·3	2·7	2·5

be seen that the responses to noradrenaline after one week were markedly higher than those after one day. It may be added that in cat 1 the superior cervical ganglion was later extirpated. This caused a sensitization similar to that seen one week after guanethidine 150 mg/kg; noradrenaline 10 μg/kg caused a secretion of 6.7 drops one week after the drug and 6.5 drops one week after ganglionectomy.

The slow elimination of the drugs is reflected in the long-lasting sensitization, as shown in the table. This was apparent in another experiment also ; two weeks after discontinuation of daily treatment with bretylium 10 mg/kg for one week, stimulation of the exposed sympathetic trunk was found to cause no secretion of saliva.

DISCUSSION

The results of the experiments described above indicate that treatment with bretylium or guanethidine for some time causes a supersensitivity similar to that created by removal of the superior cervical ganglion, that is, that these drugs in chronic experiments can cause a supersensitivity by producing a prolonged " pharmacological sympathectomy." The mode of action of these interesting drugs is not altogether clear, but the ultimate result of their action on the adrenergic fibres seems to be to prevent the nerve impulse from releasing the chemical transmitter. It therefore seems reasonable to quote the present experiments in support of the view that supersensitivity following extirpation of the superior cervical ganglion develops as a consequence of reduced liberation of transmitter substance. Since section of the preganglionic sympathetic fibres causes no supersensitivity in the submaxillary gland (Emmelin & Engström, 1960b), the conclusion would be that the transmitter substance is released from the postganglionic neurone even if this neurone is decentralized, that is, independent of impulses from the central nervous system.

A similar conclusion was made from experiments on the parasympathetic nervous system showing that botulinum toxin causes a supersensitivity of the submaxillary cells resembling that brought about by parasympathetic denervation (Emmelin, 1961b).

Bretylium tosylate was kindly supplied by Mr. A. F. Green, of the Wellcome Research Laboratories, and guanethidine by Ciba A.G.

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EFFECT OF NORADRENALINE, BRETYLIUM AND COCAINE ON THE BLOOD PRESSURE RESPONSE TO TYRAMINE IN THE RAT

BY

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Tyramine is mainly a pressor agent in the rat under urethane, although the hypertensive effect of tyramine in some experiments is followed by a prolonged increase or depression of blood pressure. Bretylium, in doses up to 10 mg/kg, prolonged the response to tyramine, whereas larger doses depressed or blocked its effect. When the hypertensive effect of tyramine was blocked by bretylium, both noradrenaline and dihydroxyphenylalanine, when slowly infused, were found to restore it. The well-known block by cocaine of the hypertensive response to tyramine could also be reversed by intravenous infusion of noradrenaline and dihydroxyphenylalanine. It is concluded that the infusion of noradrenaline and dihydroxyphenylalanine makes available noradrenaline in the postganglionic adrenergic nerves which is necessary for the action of tyramine.

In spinal preparations made from cats previously treated with reserpine, tyramine and many other sympathomimetic amines which are not derivatives of catechol lose their pressor action. This action can be restored in the reserpine-treated animal by an infusion of noradrenaline (Burn & Rand, 1958a). These observations suggest that tyramine and similar substances normally act by releasing noradrenaline or adrenaline from the arterial walls. In fact it has been shown that under certain conditions intravenous injections of tyramine increase the concentrations of adrenaline and noradrenaline in a sample of plasma withdrawn from the lower aortae of cats under chloralose.

Tyramine is also classified as a sympathomimetic substance acting on postganglionic adrenergic nerve fibres because it does not act on denervated effector organs (Burn & Tainter, 1931 ; Fleckenstein & Burn, 1953). Bretylium is found to depress conduction of impulses in adrenergic neurones with subsequent failure of noradrenaline and adrenaline release, leaving at the same time intact the adrenergic receptors of the effector cell (Boura & Green, 1959). It was therefore of interest to investigate the effect of bretylium on the vascular response to tyramine. It was expected that the effect of tyramine would be particularly easy to block if this substance acted primarily on the postganglionic adrenergic fibres. This work was started in order to investigate the site and mechanism of action of tyramine in causing changes of blood pressure in the rat. The experiments with bretylium were compared with the results obtained with cocaine in order to show their similar modes of action.

METHODS

Rats of both sexes (145 to 300 g) were used and anaesthetized with 0.7 ml. per 100 g body weight 25% urethane solution subcutaneously. To record the blood pressure a cannula was inserted into the carotid artery and connected with a mercury manometer. Drugs were injected via a small polythene cannula, 0.5 mm in diameter, which was inserted into the jugular vein. Before the experiment was started, 1 to 1.5 mg/100 g heparin was injected. All doses of drugs were injected in a vol. of 0.1 ml. and washed in with the same vol. of 0.9% sodium chloride solution.

The slow intravenous infusion of noradrenaline and dihydroxyphenylalanine was given from a microburette which was connected with the venous cannula. The rate of infusion ranged from 0.66 to 3 μg/min for noradrenaline, and from 0.16 to 0.3 mg/min for dihydroxyphenylalanine.

The following substances were used: noradrenaline bitartrate, tyramine hydrochloride, bretylium tosylate and cocaine hydrochloride. Doses referred to in this paper are calculated as the salts.

RESULTS

The effects of tyramine, noradrenaline and dihydroxyphenylalanine. It was found that tyramine always caused a rise of blood pressure similar to that caused by noradrenaline or adrenaline. In some experiments tyramine caused a biphasic effect in which an initial short-lasting hypertension was succeeded by a prolonged

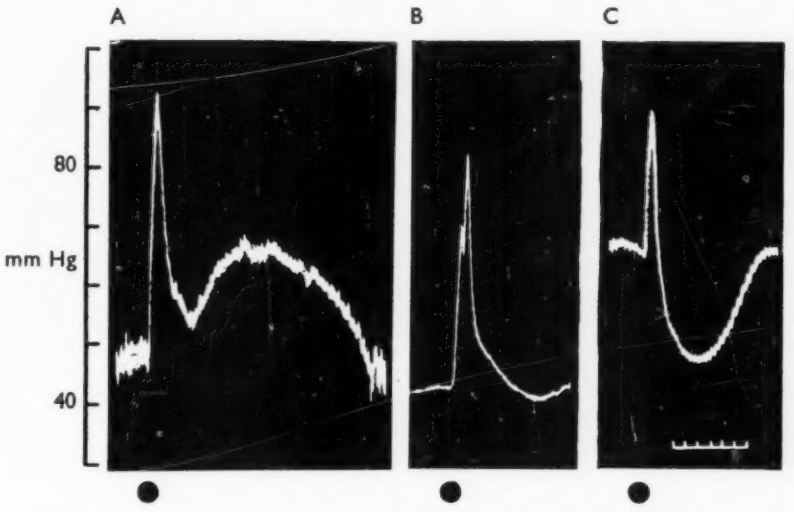

Fig. 1. Rat under urethane. Various responses of the blood pressure to the intravenous injection of 100 μg tyramine.

secondary hypertension (in 8 out of 22 experiments) or hypotension (7 out of 22). The various types of blood pressure response to tyramine in the rat are shown in Fig. 1.

The repeated injections of small doses of tyramine caused a gradual decrease in response. Fig. 2 shows an experiment in which 40 μg tyramine was injected every 10 min. After seven injections the response to tyramine had decreased from the effect shown in A to that in B. Between B and C 20 μg noradrenaline was slowly

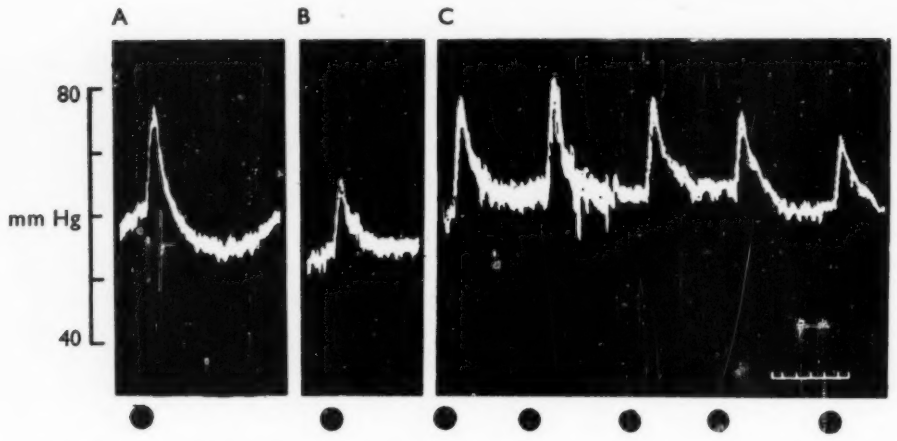

Fig. 2. Rat (190 g) under urethane. The effect of intravenous infusion of noradrenaline on the response of the blood pressure to intravenous injections of 40 μg tyramine (at dots). B, 70 min after A. Between B and C, 20 μg noradrenaline was infused over 10 min. C was taken after the infusion of noradrenaline had been stopped.

infused intravenously over 10 min. After the infusion of noradrenaline had been stopped and after the blood pressure rise caused by it had subsided, the injection of tyramine caused an increased response, as shown in C. This potentiation gradually wore off in 35 min, as also shown in C.

The infusion of dihydroxyphenylalanine produced the same effect as noradrenaline. This finding is in agreement with the finding of Burn & Rand (1960a) that the pressor action of tyramine in cats and rats treated with reserpine could be restored by intravenous infusion of various precursors of noradrenaline, for example, dihydroxyphenylalanine, dopamine, *m*-tyrosine and phenylalanine. We have now shown that even in the normal rat dihydroxyphenylalanine and noradrenaline potentiate the blood pressure response to tyramine. It was also observed that the response to tyramine after infusion of noradrenaline or dihydroxyphenylalanine was changed from a biphasic one to a pure hypertension.

The effects of tyramine, bretylium, noradrenaline and dihydroxyphenylalanine. The effect of bretylium varied according to the dose. After a dose of 10 mg/kg the response to tyramine was regularly prolonged (in 22 out of 24 experiments). Treatment of the animal by 20 mg/kg bretylium or more caused a depression or even abolition of the effect of tyramine. After such a depression the response to tyramine could be restored by an intravenous infusion of noradrenaline. A typical experiment is shown in Fig. 3. Between A and B 10 mg/kg bretylium was injected, and 15 min later the response to tyramine was prolonged, as shown in B. Between B and C 20 mg/kg bretylium was injected and 20 min later the effect of tyramine was almost abolished, as shown in C. Between C and D 30 μg noradrenaline was slowly infused intravenously over 15 min. After the infusion of noradrenaline had been stopped and after the blood pressure rise caused by it had subsided, the injection of tyramine caused an increased response, as shown in D. This type of response was observed in 6 out of 8 experiments.

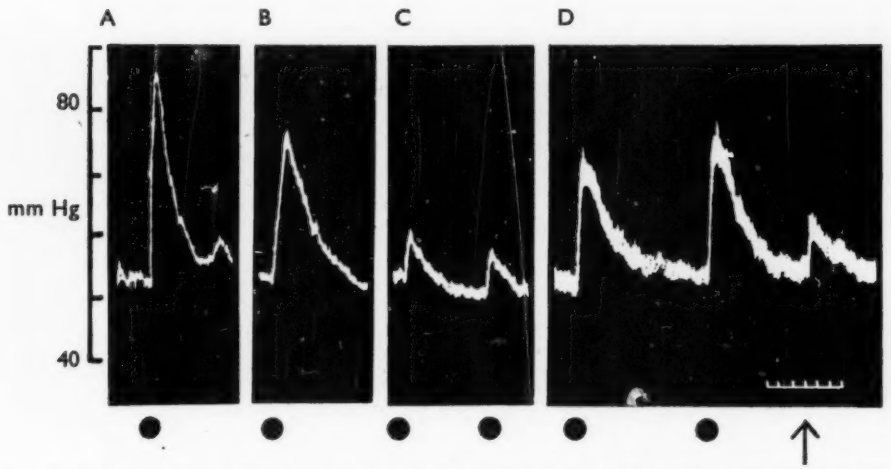

Fig. 3. Rat (155 g) under urethane. Injections into the jugular vein. The effect of bretylium and noradrenaline on the blood pressure response to tyramine. Between A and B, 10 mg/kg bretylium. Between B and C, 20 mg/kg bretylium. Between C and D, slow infusion of 30 μg noradrenaline. At dots, 50 μg tyramine; at the arrow, 0.1 ml. 0.9% sodium chloride solution.

Similar effects were obtained after infusion of dihydroxyphenylalanine. Fig. 4 shows an experiment in which 22 mg/kg bretylium abolished almost completely the effect of tyramine, as shown in B. Between B and C 3 mg dihydroxyphenylalanine was slowly infused intravenously over 15 min. After the infusion had been stopped the hypertensive response to tyramine was restored, as shown in C. This type of response was observed in 4 out of 5 experiments.

The effects of tyramine, cocaine, noradrenaline and dihydroxyphenylalanine. It is well known that cocaine can block the effect of tyramine (Burn & Tainter, 1931). We have now shown that the response to tyramine, once it was blocked by cocaine, could

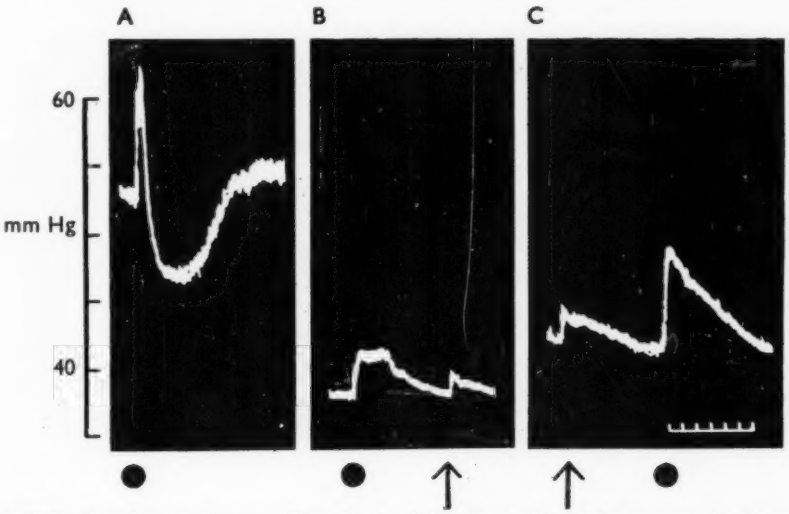

Fig. 4. Rat (170 g) under urethane. The effect of dihydroxyphenylalanine on the blood pressure response to tyramine (50 μg, at dots). Between A and B, 22 mg/kg bretylium. Between B and C, slow intravenous infusion of 3 mg dihydroxyphenylalanine. At the arrows, 0.1 ml. 0.9% sodium chloride solution.

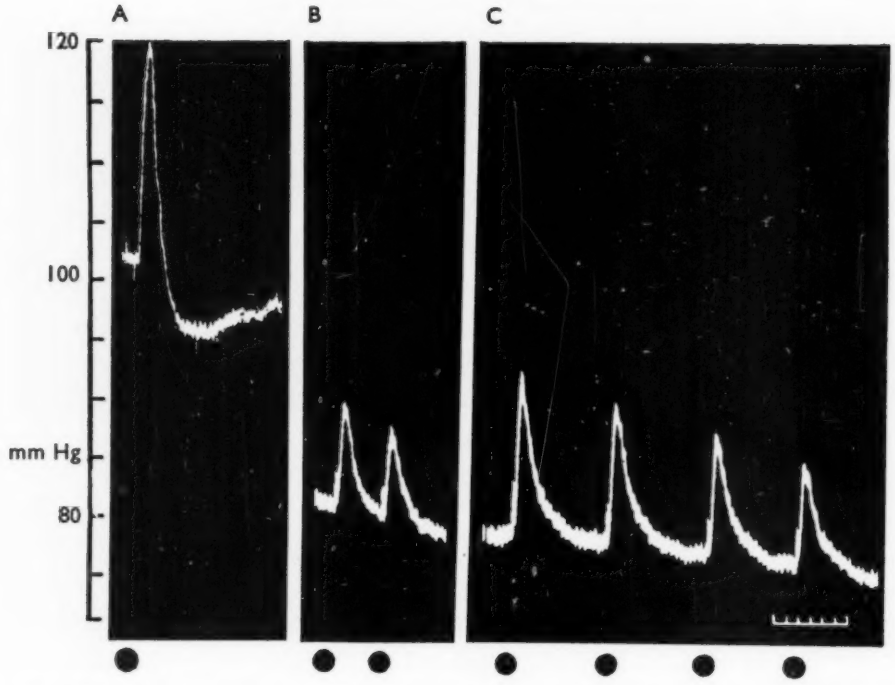

Fig. 5. Rat (210 g) under urethane. The effect of cocaine and noradrenaline on the blood pressure response to tyramine (200 μg, at dots). Between A and B, 0.5 mg cocaine. Between B and C, slow intravenous infusion of 15 μg noradrenaline.

be restored by an intravenous infusion of noradrenaline. A typical experiment is shown in Fig. 5. Between A and B 2.5 mg/kg cocaine was injected intravenously, and 20 min later the response to tyramine was very much depressed, as shown in B. Between B and C 15 μg noradrenaline was slowly infused over 15 min. After the infusion had been stopped and when the rise of blood pressure caused by it had subsided, the injection of tyramine produced a greater response than before the infusion, as shown in C. This type of response was observed in 5 out of 9 experiments.

In 2 out of 3 experiments the intravenous infusion of dihydroxyphenylalanine in animals treated with cocaine caused an increase of the hypertensive response to tyramine. This effect of dihydroxyphenylalanine was generally observed if dihydroxyphenylalanine by itself produced a small but long-lasting increase in blood pressure.

DISCUSSION

The present experiments show that tyramine is mainly a pressor agent in the rat under urethane. In some experiments this initial hypertension is followed by a prolonged increase or depression of blood pressure. The secondary fall of blood pressure caused by tyramine was similar to the blood pressure fall caused by dopamine in the guinea-pig (Hornykiewicz, 1958 ; Burn & Rand, 1958b). It was also found that the hypertensive effect of tyramine gradually diminished after repeated injections. This tachyphylaxis could be immediately reversed after infusion of noradrenaline and dihydroxyphenylalanine.

It was found in previous work that bretylium blocked the hypertensive effect of eserine in the rat, leaving at the same time intact or even potentiating the effect of noradrenaline (Lešić & Varagić, 1961). We have now found that the hypertensive effect of tyramine is usually influenced in a similar way. However, larger doses of bretylium were necessary to depress the effect of tyramine than to block the effect of eserine. The doses of bretylium up to 10 mg/kg caused a prolongation of the response to tyramine. Burn & Rand (1960b) have already observed that the effect of tyramine on the nictitating membrane of the spinal cat may be potentiated by bretylium. If the hypertensive effect of tyramine was blocked by a large dose of bretylium, both noradrenaline and dihydroxyphenylalanine, when slowly infused, were found to be able to restore that effect. Bretylium is known to impair conduction of impulses in adrenergic neurones with consequent failure of release of noradrenaline and adrenaline (Boura & Green, 1959). Tyramine, on the other hand, is known to liberate noradrenaline from adrenergic transmitter granules (von Euler & Lishajko, 1960 ; Schümann, 1960) and from the walls of the aorta in cats under chloralose after induction of lasting ganglion block with hexamethonium and after exclusion of the adrenals from the circulation (Lockett & Eakins, 1960a & b). In postganglionically denervated sympathetically innervated effector organs, as well as in animals treated with cocaine, the effect of tyramine was abolished (Burn & Tainter, 1931 ; Fleckenstein & Burn, 1953 ; Fleckenstein & Stöckle, 1955). In animals treated with reserpine the effect of tyramine was also abolished (Carlsson, Rosengren, Bertler & Nilsson, 1957). All the evidence suggested that tyramine acted through the liberation of noradrenaline (Burn & Rand, 1958a). Our experiments support the idea that the infusion of noradrenaline and dihydroxyphenylalanine in animals treated with bretylium restores the stores of noradrenaline in postganglionic adrenergic nerves. These stores are presumably necessary for the action of tyramine.

The indirect effects of sympathomimetic amines (for example, tyramine) which can be abolished by reserpine are decreased by cocaine, which presumably acts by inhibiting the liberation of noradrenaline (Holtz, Osswald & Stock, 1960). The present experiments show that the block by cocaine of the hypertensive response to tyramine could be reversed by intravenous infusion of noradrenaline and dihydroxyphenylalanine. This finding seems to suggest that "pharmacological denervation" (Fleckenstein & Stöckle, 1955) is due to impairment of noradrenaline release from postganglionic adrenergic nerves. The infusion of noradrenaline and dihydroxyphenylalanine makes available noradrenaline in the postganglionic adrenergic nerves which is necessary for the action of tyramine.

Until recently the fate of the infused noradrenaline and adrenaline was an unsolved problem. Thus Raab & Gigee (1955) observed that the heart muscle of the dog took up noradrenaline and adrenaline after intraperitoneal injection of these substances. It may be noted that the amounts of catecholamines in these experiments were very high (38 mg noradrenaline and 75 mg adrenaline for a dog of 10 kg). Von Euler (1956) infused amounts of noradrenaline between 2.5 and 7.8 μg/kg/min into cats for over 30 min and gave intraperitoneal injections of noradrenaline and adrenaline up to 2 mg/kg. In these experiments no increase of catechol amine content of the heart, spleen, liver, kidney and skeletal muscle was

observed. In experiments with tritium-labelled ^{3}H-adrenaline of high specific activity, it was found that immediately after the end of a 30 min infusion (3 μg/kg/min) the concentration of ^{3}H-adrenaline in heart, spleen, adrenal and pituitary gland exceeded that of the plasma by several-fold. Even two hours after the administration of ^{3}H-adrenaline large quantities were found in the heart and spleen, indicating that these tissues not only accumulate adrenaline but also retain it for a long period (Axelrod, Weil-Malherbe & Tomchick, 1959a & b). Our experiments suggest that the infused noradrenaline and its precursor, dihydroxyphenylalanine, are partly accumulating in the adrenergic nerve terminals Von Euler (1956) has already expressed the view that, at least in the spleen, noradrenaline is present within the fine terminations of the nerves.

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Brit. J. Pharmacol. (1961), **16**, 327–334.

POTENTIATION OF INHIBITORY AND EXCITATORY EFFECTS OF CATECHOL AMINES BY BRETYLIUM

BY

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Enhancement of both excitatory and inhibitory responses to adrenaline, noradrenaline and isoprenaline was demonstrated with bretylium both *in vivo* and *in vitro*. Excitatory responses studied included rise of blood pressure and contraction of the nictitating membrane in cats and the contraction of aortic strip in rabbits; the inhibitory responses studied were fall of cat blood pressure and relaxation of rat uterus and rabbit tracheal chain. Possible mechanisms of potentiation of the effects of catechol amines by bretylium are discussed. A sensitization of the peripheral effector cells is suggested as a likely mechanism of potentiation.

A new hypotensive drug, bretylium, has recently been introduced in therapeutics. Pharmacological tests on animals have shown that it blocks adrenergic neurones, without antagonizing the effects of released or injected adrenaline or noradrenaline (Boura & Green, 1959). Studying the effects of bretylium on cardiovascular control in dogs, Aviado & Dil (1960) reported that after the injection of bretylium the pressor response induced by noradrenaline was enhanced and prolonged. Vernikos-Danellis & Zaimis (1960) have also reported a potentiation of the blood pressure effects of adrenaline and noradrenaline after pretreatment with bretylium. The final explanation for the augmentation of the effects of these drugs by bretylium is still not certain. One possibility is that the effector cells in heart and blood vessels are sensitized to adrenaline and noradrenaline. Another possibility is that bretylium blocks the compensatory reflexes that are known to be excited by any pressor agent. If bretylium is shown to have a peripheral action in the form of a sensitization of the effector cells to adrenaline and noradrenaline, it might help to explain the " tolerance " that is so often reported with bretylium in clinical practice.

An attempt is made to characterize the potentiation of the blood pressure effect of adrenaline, noradrenaline and isoprenaline by bretylium. The effect of bretylium on other excitatory and inhibitory responses to adrenaline, noradrenaline and isoprenaline is also studied.

METHODS

Cats. The effect of bretylium on blood pressure and nictitating membrane responses to noradrenaline, adrenaline and isoprenaline was studied in intact and in vagotomized cats anaesthetized with chloralose 80 mg/kg and in spinal vagotomized cats. Blood pressure was recorded from a cannula in the left common carotid artery. The nictitating membrane of

the right side was connected to a frontal writing lever and isotonic contractions were recorded on a smoked paper. All drugs were dissolved in normal saline and injected through a polyethylene cannula in the femoral vein.

Fixed doses of noradrenaline, adrenaline or isoprenaline were administered rapidly at 10 min intervals. When the response produced by a fixed dose was reproducible, bretylium was administered in a dose of 5 mg/kg; the catechol amine was again administered 15 min after injection of bretylium and at 10 min intervals for 1 hr. The following measurements were made of the responses to adrenaline, noradrenaline and isoprenaline: △ B.P.—the difference between the control systolic blood pressure and peak systolic pressure of the pressor or depressor response expressed in mm of Hg; decay time 50 (DT 50): the time in sec from the onset of the pressor or depressor response to the time when the response had decreased to one-half of the maximum.

Aortic strips. Strips were obtained from the thoracic aorta of young rabbits and prepared in the manner described by Furchgott & Bhadrakom (1953). Spirally cut aortic strips approximately 3.5 cm in length were placed in a 50 ml. organ bath and tied to an isotonic frontal writing lever that placed the muscle under 5 g of tension at the horizontal equilibrium position. The bathing solution was Krebs bicarbonate solution containing 0.01 M glucose. The solution was maintained at 37 to 38° C, and 5% carbon dioxide in oxygen was bubbled through the solution in both bath and reservoir. The preparation was left for 2 hr before testing.

Dose-response curves for noradrenaline and adrenaline were obtained by cumulative administration of increasing concentrations of the amines, allowing the contraction to develop fully after each administration. Dose-response relationships for noradrenaline and adrenaline were determined in replicate on each strip prior to administration of bretylium. In studies on potentiation, bretylium was placed in the bath 5 min before administration of catechol amines and remained in the bath thereafter.

Tracheal chains. Rabbit tracheal chains were prepared according to the method of Castillo & deBeer (1947). Preparations were suspended in a 50 ml. organ bath and connected to a balsa-wood frontal writing lever. Krebs bicarbonate solution containing 0.01 M glucose and aerated with 95% oxygen and 5% carbon dioxide was used as the bathing medium. Pilocarpine was added in a concentration of 1 mg/100 ml. The responses to adrenaline and isoprenaline were recorded on smoked kymograph paper after the pilocarpine-induced spasm had reached a stable plateau.

Rat uterus. Uterine horns of non-pregnant rats were suspended in a 30 ml. bath and connected to a frontal writing lever. De Jalon fluid aerated with 95% oxygen and 5% carbon dioxide and maintained at 30° C was used as the bathing medium. Reduction of acetylcholine-induced contractions of the rat uterus was taken as a measure of the inhibitory effect of adrenaline and isoprenaline. When the inhibition produced by a fixed dose was constant, bretylium was added to the bath and allowed to act for 30 sec; the inhibitory effect of the amines was again determined.

Drugs. Bretylium was used as bretylium tosylate, and the doses or concentrations refer to the salt. Adrenaline and noradrenaline were used as the base dissolved in normal saline and the doses or concentrations refer to the base. Isoprenaline was used as isoprenaline sulphate and the doses or concentrations refer to the salt.

RESULTS

Effect of bretylium on cat blood pressure and nictitating membrane responses to catechol amines. Moderate doses of bretylium (5 to 7 mg/kg) administered rapidly intravenously always produced a biphasic response in blood pressure in all the preparations. There was a brief initial fall followed by a rise which persisted for

TABLE 1

EFFECT OF BRETYLIUM (5 MG/KG) ON MAGNITUDE AND DURATION OF BLOOD PRESSURE RESPONSES TO ADRENALINE, NORADRENALINE AND ISOPRENALINE

$$\% = \frac{\text{Value in treated animal}}{\text{Value in untreated animal}} \times 100$$

	Adrenaline (0·5 to 2·0 μg/kg)			Noradrenaline (0·5 to 2·0 μg/kg)			Isoprenaline (0·5 to 2·0 μg/kg)		
	Type of preparation								
	Intact	Vagoto-mized	Spinal vagoto-mized	Intact	Vagoto-mized	Spinal vagoto-mized	Intact	Vagoto-mized	Spinal vagoto-mized
Untreated Δ B.P. mm of Hg	30	77	42	38	64	47	16	77	20
Treated Δ B.P. mm of Hg	48	101	98	73	90	126	27	111	36
%	160	130	230	190	140	260	170	140	190
	($P<0·01$)	($P<0·05$)	($P<0·01$)	($P<0·01$)	($P<0·01$)	($P<0·01$)	($P<0·01$)	($P<0·01$)	($P<0·05$)
Untreated DT 50 sec	69	120	90	60	97	72	34	36	66
Treated DT 50 sec	114	196	116	66	178	97	48	51	95
%	165	163	129	110	183	134	141	141	144
	($P<0·05$)	($P<0·01$)	($P>0·05$)	($P<0·01$)	($P<0·01$)	($P<0·01$)	($P<0·01$)	($P<0·01$)	($P<0·01$)

10 to 12 min. During the later portion and the peak of the pressor response there was an increase in pulse pressure. As a rule blood pressure stabilized at or slightly above control blood pressure within 15 min.

Table 1 summarizes the changes produced by bretylium in the vasomotor reactivity of intact, vagotomized and spinal vagotomized cats to fixed doses of adrenaline, noradrenaline and isoprenaline (0.5 to 2.0 μg/kg). The control values are means of 3 to 8 observations made in 2 or 3 animals, while the values after treatment with bretylium represent the mean of 8 to 14 observations made at 10 min intervals in 3 to 4 cats for 1 hr following the administration of bretylium.

Doses of 5 to 7 mg/kg of bretylium produced a simultaneous potentiation of the pressor effect and of the response of the nictitating membrane to adrenaline and noradrenaline in intact, vagotomized and spinal vagotomized cats. It also potentiated the depressor effect of isoprenaline (Figs. 1 and 2).

The potentiating effect of bretylium on the blood pressure responses to the catechol amines was best seen in the spinal vagotomized preparation, the magnitude of the responses to adrenaline, noradrenaline and isoprenaline being enhanced to 230, 260 and 190% respectively of the control values. Bretylium also prolonged the mean duration of the pressor or depressor response to the catechol amines. The effect was more significant for noradrenaline and isoprenaline than for adrenaline.

Aortic strips. The major difficulty of *in vitro* investigation of the action of sympathomimetic amines has been the selection of a suitable biological test preparation. Furchgott (1955) has shown that the aortic strip preparation fulfils many of

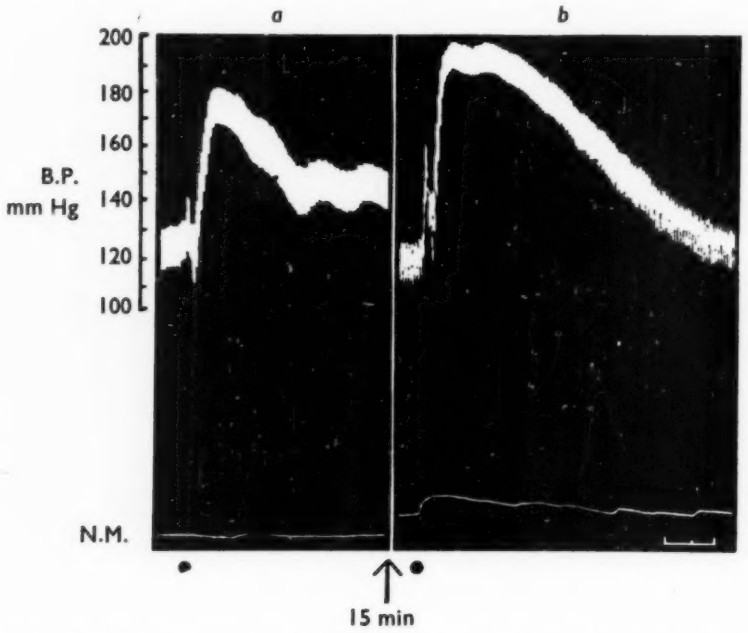

Fig. 1. Cat, 3 kg, chloralose anaesthesia. Both vagi cut in the midcervical region. Records of carotid arterial blood pressure (B.P.) and nictitating membrane (N.M.). Responses to intravenous injections of 6 μg of noradrenaline (at the dots) before (*a*) and 15 min after 5 mg/kg bretylium (*b*). Time, 1 min.

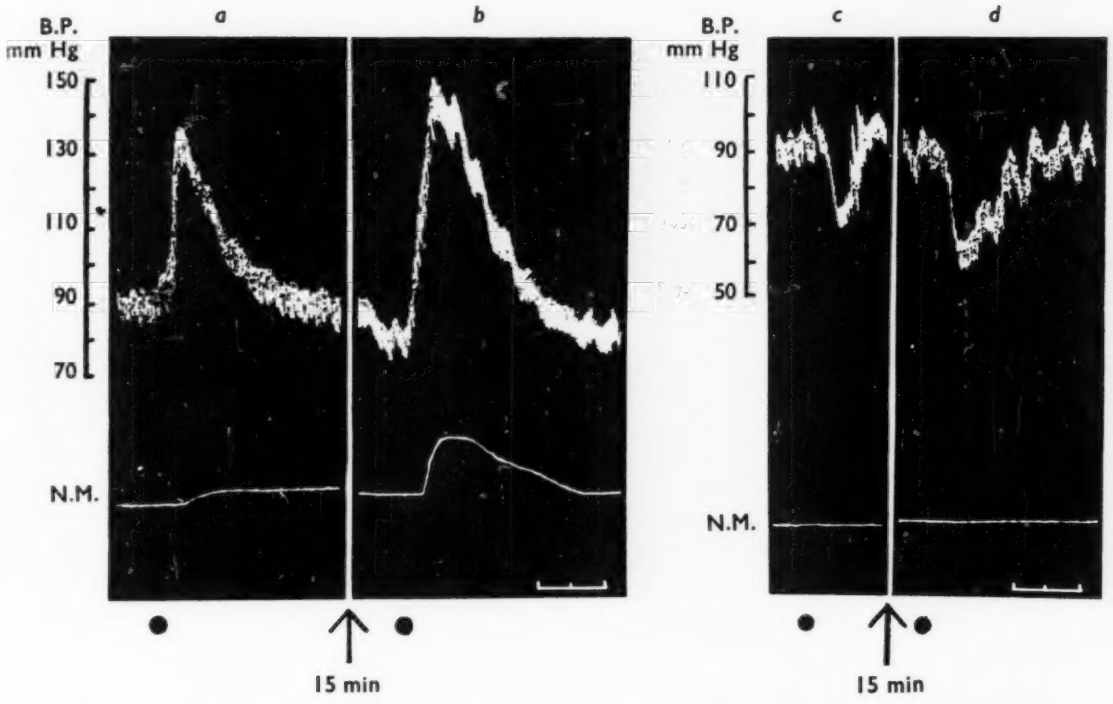

Fig. 2. Cat, 2.6 kg, spinal vagotomized preparation. Records of carotid arterial blood pressure (B.P.) and nictitating membrane (N.M.). Responses to intravenous injections (at the dots) of 5 μg adrenaline (*a* and *b*) and of 5 μg isoprenaline (*c* and *d*) before (*a* and *c*) and 15 min after (*b* and *d*) 5 mg/kg bretylium. Time, 1 min.

the requirements of an ideal test object. It contains only one type of pharmacologically active cell, and after an initial equilibration in saline it shows no residual tone and no spontaneous activity. This preparation was therefore chosen to study the effect of bretylium on the excitatory responses of adrenaline and noradrenaline *in vitro*.

A concentration of 2×10^{-5} of bretylium potentiated the contractions induced by adrenaline and noradrenaline (Table 2). In Fig. 3 the responses to adrenaline and

TABLE 2

ENHANCEMENT BY BRETYLIUM OF EXCITATORY AND INHIBITORY RESPONSES TO CATECHOL AMINES

— indicates no detectable response

Preparation	No. of experiments	Dose or concentration of bretylium	Average % control response		
			Noradrenaline	Adrenaline	Isoprenaline
Nictitating membrane	5	5 mg/kg	250	210	—
Aortic strips	6	2×10^{-5}	155	116	—
Rat uterus	6	2×10^{-6}	—	144	—
	6	5×10^{-6}	—	165	—
	6	1×10^{-5}	—	177	134
	7	2×10^{-5}	—	221	154
Tracheal chain	6	5×10^{-6}	—	183	120
	6	1×10^{-5}	—	300	135
	6	2×10^{-5}	—	500	150

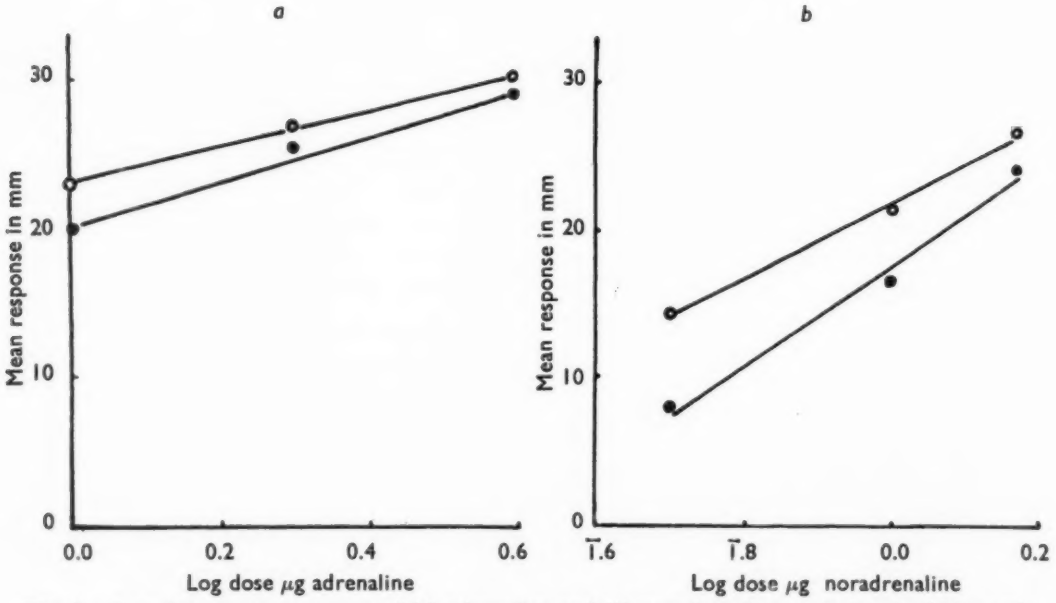

Fig. 3. Log dose-response curves of (*a*) adrenaline and (*b*) noradrenaline on isolated rabbit aortic strips, alone (closed circles) and in the presence of bretylium (open circles).

noradrenaline are plotted on a graph to give the dose-response curves, alone and in the presence of bretylium. The potentiation is more pronounced with lower doses of the amines and decreases with higher doses.

Tracheal chain. The rabbit tracheal chain was used to study the effect of bretylium on inhibitory responses to adrenaline and isoprenaline. The responses of the preparation to adrenaline and isoprenaline varied with repeated doses ; the addition of pilocarpine to the bathing fluid enabled a uniform inhibitory response to be obtained.

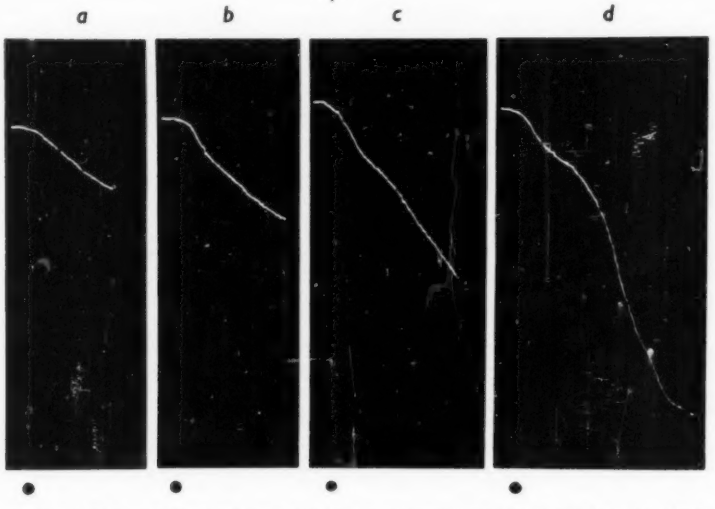

Fig. 4. Responses of the rabbit tracheal chain to adrenaline 1×10^{-6} (at the dots) alone (*a*) and in the presence of bretylium 5×10^{-6} (*b*), bretylium 1×10^{-5} (*c*), and bretylium 2×10^{-5} (*d*). Contact time, 2 min.

Bretylium in concentrations of 5×10^{-6}, 1×10^{-5} and 2×10^{-5} did not alter the pilocarpine-induced spasm, but after exposure for 1 min the relaxing effect of adrenaline or isoprenaline was considerably enhanced (Table 2). Fig. 4 shows the potentiation of the relaxant effect of adrenaline (1×10^{-6}) by increasing concentrations of bretylium. The potentiation was related to the dose.

Rat uterus. The most sensitive biological method of estimating adrenaline is by its inhibition of acetylcholine-induced contractions of the rat uterus. The percentage reduction was determined before and in the presence of various concentrations of bretylium. Bretylium concentrations of 2×10^{-5}, 1×10^{-5}, 5×10^{-6} and 2×10^{-6} greatly enhanced the inhibitory action of adrenaline or isoprenaline (0.6×10^{-9}) on the rat uterus (Table 2). Fig. 5 shows the inhibitory action of

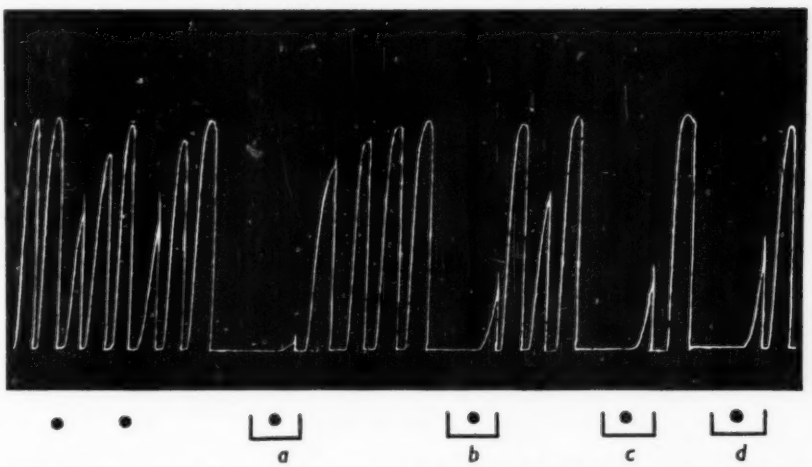

Fig. 5. Inhibitory action of adrenaline 0.6×10^{-9} (added at the dots) on acetylcholine (0.3×10^{-6}) induced contractions of the rat uterus. Contact time 30 sec for both acetylcholine and adrenaline. The period marked by bar (*a*) indicates the presence of bretylium 2×10^{-5}, (*b*) bretylium 1×10^{-5}, (*c*) bretylium 5×10^{-6}, and (*d*) bretylium 2×10^{-6}.

0.6×10^{-9} of adrenaline before and in the presence of varying amounts of bretylium. The potentiation produced by bretylium is related to the dose. Bretylium in the highest concentration used, that is, 2×10^{-5}, did not affect acetylcholine-induced contractions.

DISCUSSION

The present study has shown that bretylium enhances *in vivo* the magnitude and duration of certain excitatory and inhibitory effects of the three catechol amines employed, adrenaline, noradrenaline and isoprenaline. This potentiation could not be ascribed to the suppression by bretylium of the baroreceptive reflexes, since potentiation is obtainable in bilaterally vagotomized animals and spinal vagotomized cats. Further, Aviado & Dil (1960) have reported that bretylium alone could not block the increase of blood pressure due to the carotid sinus reflex elicited by bilateral clamping of the common carotid arteries. A combination of adrenalectomy and bretylium was required to abolish the carotid sinus reflex. Moreover, the dose of bretylium required (20 mg/kg) was far too high. Thus a blockade of the

compensatory cardiovascular reflexes by bretylium can be eliminated as a cause of potentiation.

From studies of the effects of sympathomimetic amines in normal and reserpine pretreated animals, Burn & Rand (1958) have proposed that ephedrine and other non-catechol compounds of this class act primarily by releasing a noradrenaline-like substance from available stores in the vascular walls. In terms of this concept the heightened response to catechol amines immediately following a dose of ephedrine might be attributed to temporary depletion of the stores and a consequent availability of a larger proportion of the receptor sites in the vascular smooth muscle to injected catechol amines. A similar mechanism for the action of bretylium in potentiating the effects of catechol amines is not excluded by our results.

Mantegazza, Tyler & Zaimis (1958) have reported that the ganglion-blocking agents, hexamethonium and pentolinium, enhance the responses of the nictitating membrane and peripheral blood vessels to injected catechol amines. This action was attributed to sensitization of the effector cells, possibly through changes in permeability. A similar explanation could be suggested for the potentiation of the catechol amines by bretylium. We have been able to demonstrate enhancement of the action of the catechol amines *in vitro* by bretylium. This potentiation of the *in vitro* effects of the catecholamines by bretylium indicates sensitization of the effector cells as the mechanism of potentiation.

Thus sensitization of the effector cells in the heart and blood vessels may well account for the increase in pressor response produced by adrenaline or noradrenaline in presence of bretylium, and for the development of tolerance which appears during the treatment of hypertensive patients.

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EFFECT OF NORADRENALINE ON THE ACTION OF NICOTINE AND TYRAMINE ON ISOLATED ATRIA

BY

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Both nicotine (in the presence of atropine) and tyramine cause a rise in the rate of isolated rabbit atria. When noradrenaline is allowed to act on the atria for 20 to 30 min and then removed from the bath by repeated changes of the bath fluid, the action of nicotine and of tyramine is greatly increased. The first addition of noradrenaline to the bath often has a much smaller effect on the response to nicotine or to tyramine than have later additions. Sometimes the greater effect after the addition of nicotine persists for 2 or 3 hr. When reserpine is added to the bath and left in contact with the atria for 1 to 2 hr, and is then removed from the bath, the effect of noradrenaline on the atrial rate is much reduced in size and duration. Bretylium abolishes the action of nicotine but increases the action of tyramine.

The first suggestion that noradrenaline, when infused slowly into the blood stream, was taken up by tissues with a sympathetic innervation was made by Burn & Rand (1958). They observed that when the pressor and constrictor action of tyramine, and the constrictor action of sympathetic stimulation, were absent in cats and dogs previously treated with reserpine, these actions could be restored by the infusion of noradrenaline, and could be observed after the infusion of noradrenaline had ceased and its direct effect had passed away. They also observed that in normal animals which had not been treated with reserpine the constrictor action of tyramine and of sympathetic stimulation was increased after an infusion of noradrenaline (Burn & Rand, 1960a). Positive evidence of the uptake of noradrenaline was obtained by Pennefather & Rand (1960), who observed that after an infusion of noradrenaline the kidney and the horn of the uterus of one side each contained more noradrenaline than the organ of the opposite side removed before the infusion began. Positive evidence was also obtained by Whitby, Hertting & Axelrod (1960), who injected DL-β-^{3}H noradrenaline into cats intravenously, and saw that the ^{3}H noradrenaline was taken up by the adrenal gland, heart and spleen when these tissues were examined 1 hr after the injection of the noradrenaline. Small amounts were also taken up by the liver and muscle. Evidence has also been obtained by Muscholl (1960) that infusion of noradrenaline into the spinal rat increased the content of noradrenaline in the heart.

Evidence of uptake has also been obtained in isolated tissues. Gillespie & Mackenna (1959) suspended a piece of rabbit colon in a bath together with the

sympathetic nerves. Stimulation of these nerves caused inhibition of the contractions. When, however, the preparation was made from a rabbit which had been treated with reserpine, stimulation of the sympathetic nerves caused contraction of the colon. When noradrenaline was added to the bath and allowed to remain there for 15 min, stimulation of the nerves once more produced its usual inhibitory effect. This observation suggested that even in an organ bath the tissue could still take up noradrenaline, and hold it in such a way that it could be released by stimulation of sympathetic nerves. Huković (1961) has observed the contractions of the isolated vas deferens in response to hypogastric stimulation, and has seen that the contractions were increased after noradrenaline was added to the bath for 30 min and then washed out.

Observations have now been made on isolated atria from normal rabbits, not treated with reserpine, to study the effect of noradrenaline on the action of nicotine and of tyramine in causing an increase of atrial rate.

METHODS

The heart was excised from a freshly killed rabbit of about 2 kg. The atria were dissected at room temperature in a solution of the following composition, which was also used in the isolated organ bath: sodium chloride 8.5 g, potassium chloride 0.42 g, calcium chloride 0.24 g, dextrose 2.0 g, sodium bicarbonate 0.5 g, distilled water to 1 litre. This solution was bubbled with 95% oxygen and 5% carbon dioxide. The atria were attached to a light metal lever kept horizontal by a spring suspended vertically above the lever. When the atria contracted, a silver wire attached to the lever dipped into a salt solution in a small perspex bath and completed an electrical circuit. The current was supplied by two 45-volt batteries in series with a Post Office counter. Thus the atrial rate was recorded automatically on the counter. The organ bath was made of double-walled perspex and the atria were fully immersed in 5 ml. of solution. A stream of water at 32° C was maintained through the jacket surrounding the inner compartment. The solution was vigorously aerated through a sintered glass capsule placed below the atria.

Nicotine was used as the acid tartrate. The dose of nicotine is given in terms of the base (one-third of the weight of the salt). Tyramine was used in the form of the hydrochloride, and the dose of tyramine is given in terms of this salt. Reserpine was taken from ampoules of Serpasil supplied by Ciba. Bretylium was used in the form of bromide, and the dose is given in terms of this salt. Noradrenaline was used as L-arterenol bitartrate, and the dose is expressed in terms of the base. All observations made with nicotine were made in the presence of atropine sulphate (1 μg/ml.), which was added to the bath at least 1 min before the nicotine. The rate of beating was determined several times during a period of 5 or 10 min before nicotine or tyramine was added. When they were added, the rate was determined during six half-minute periods beginning at 30 sec, 90 sec, etc., after which the bath fluid was changed. The effect was recorded as the maximum increase in rate over the initial rate.

RESULTS

Effect of noradrenaline on the response to nicotine. In determining the effect of adding noradrenaline to the bath, a series of observations was first made on the effect of nicotine. Care was taken to see that the temperature at which the observations were made was approximately the same ($\pm$0.2° C), and nicotine was added to the bath at fairly long intervals. Thus, in the experiment which is illustrated in Table 1, the interval was 45 to 53 min between the first five additions of nicotine, was 29 min between the fifth and sixth, and thereafter was 35 min. When nor-

TABLE 1

EFFECT OF EXPOSING THE ATRIA TO NORADRENALINE ON THE RESPONSE OF THE ATRIAL RATE TO NICOTINE

Time in min	Substance added	Concentration of base μg/ml.	Time of action in min	Increase of atrial rate beats/min
0	Nicotine	2·2	6	17
48	Nicotine	2·2	6	0
94	Nicotine	2·2	6	0
100	Noradrenaline	1·0	25	
145	Nicotine	2·2		6
182	Nicotine	3·0		7
205	Nicotine	4·5		24
211	Noradrenaline	1·0	25	
240	Nicotine	4·5		94
246	Noradrenaline	1·0	25	
275	Nicotine	3·0	6	74
281	Noradrenaline	1·0	25	
308	Nicotine	2·2	6	70
335	Nicotine	3·0	6	10

adrenaline was added to the bath between applications of nicotine, it was always added for 25 min, and at the end of this time the bathing fluid was changed twice with an interval between the changes. There was then a period of 10 to 20 min for the direct effect of the noradrenaline to pass off.

In Table 1 it is seen that the first addition of a concentration of 2.2 μg/ml. nicotine increased the rate slightly by 17 beats per min, but that the second and third additions of this concentration had no effect. Noradrenaline (1 μg/ml.) was added to the bath for 25 min, and after it had been removed the concentration of 2.2 μg/ml. nicotine increased the rate by 6 beats. Thus noradrenaline in this trial had almost no effect. Higher concentrations of nicotine were then tested, 3 μg/ml. and 4.5 μg/ml., without further applications of noradrenaline ; they caused increases of rate of 7 and 24 beats respectively. The effect of noradrenaline was then determined on these higher concentrations. After application of noradrenaline (1 μg/ml.) the concentration of 4.5 μg/ml. nicotine caused an increase of 94 beats, the concentration of 3 μg/ml. an increase of 74 beats, and even the concentration of 2.2 μg/ml. caused an increase of 70 beats. This increased effect of nicotine was evident only for the application of nicotine made immediately after the exposure to noradrenaline, for the last application shown in Table 1 of 3 μg/ml. produced little more increase than it did when it was first tested.

A more lasting effect of noradrenaline, however, was observed in another experiment summarized in Table 2 as Expt. 2. Nicotine in a concentration of 2.7 μg/ml. caused the rate to increase in three successive applications by 62, 62, and 20 beats respectively. Noradrenaline (1 μg/ml.) was then added to the bath before each of the next 4 applications of nicotine and left in the bath for periods of 30, 60, 26, and 28 min respectively. The bath fluid was, as usual, changed twice and the rate returned to the initial value before nicotine was added. The increase in rate caused by nicotine (2.7 μg/ml.) was then 38, 90, 90, and 86 beats per min respectively. No further additions of noradrenaline were made, and at the next 4 applications of nicotine the rate increased by 90, 76, 72, and 72 beats. Thus the effect of the

noradrenaline in increasing the nicotine response persisted for more than 3 hr after the last application of noradrenaline.

Effect of noradrenaline on the response to tyramine. The addition of noradrenaline to the bath had a similar effect on the action of tyramine to that described on the action of nicotine. The results of three experiments (Nos. 4, 5 and 6) are summarized in Table 2. In Expt. 4 the first application of noradrenaline had little effect, for

TABLE 2

MEAN EFFECTS OF NICOTINE AND OF TYRAMINE ON ATRIAL RATE BEFORE AND AFTER EXPOSING THE ATRIA TO NORADRENALINE

Expt.	Substance	Concentration μg/ml.	Effect on atrial rate: Initial	Effect on atrial rate: After noradrenaline
2	Nicotine	2·7	48	90
3	Nicotine	2·2	25	82
4	Tyramine	0·5	26	90
5	Tyramine	3·0	10	72
6	Tyramine	1·0	6	54

the effect of tyramine (0.5 μg/ml.) was to cause an increase of rate of 27 beats per min (2 observations) before noradrenaline was added, and an increase of 32 beats per min after noradrenaline was added. The second and third applications of noradrenaline, however, were followed by a great increase in the action of tyramine. The rate rose by 82 and 90 beats per min. No further application of noradrenaline

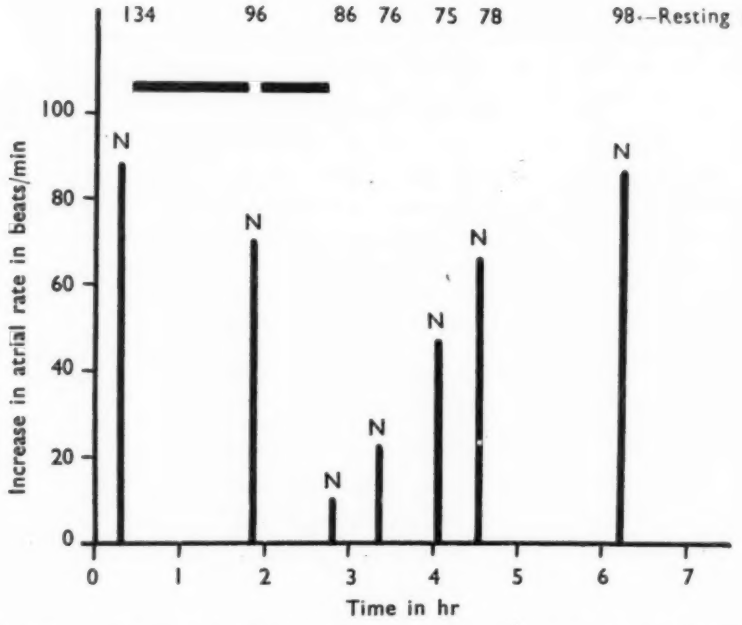

Fig. 1. The diagram shows the effect of adding reserpine to the bath on the action of nicotine. Initially nicotine (6 μg/ml.) caused the atrial rate to increase from 134 per min to 222 per min. After reserpine 4 μg/ml. had been added to the bath for the period shown by the horizontal bars, nicotine increased the rate from 86 per min to 95 per min. After changing the bath repeatedly the original effect of nicotine returned, more than 3 hr later. N=nicotine 6 μg/ml.

was made, but the next response to tyramine was still 82 beats per min. However, the next response after that was almost zero.

In two preparations the addition of tyramine caused a lasting increase in the atrial rate which persisted after tyramine was removed. In one of these the rate at the beginning was 139 per min. After the first addition of tyramine an increase of 50 beats per min was recorded. When the bath was changed to remove the tyramine, the rate remained at a higher level of 148 per min. Successive additions of tyramine caused progressively smaller increases in rate, but raised the atrial rate after the tyramine was removed from the bath. When noradrenaline was added to the bath for 43 min and then removed, the atrial rate was 192 per min. Although it was so high, the addition of tyramine raised the rate by 46 beats per min.

Effect of reserpine. We tested the effect of adding reserpine to the bath, and found that, as Fig. 1 shows, it blocked the action of nicotine in causing a large increase in the atrial rate. Reserpine was added to the bath in a concentration of 4 μg/ml. Fig. 1 shows that after reserpine was present in the bath for 82 min the effect of nicotine was slightly reduced, but that after it was added again in the same concentration for a further period of 47 min the effect of nicotine was then almost abolished. However, successive additions of nicotine (in the absence of added reserpine) were followed by a gradual return of the full nicotine effect seen at the beginning. Reserpine also reduced the atrial rate from an initial figure of 134 beats per min to 75 beats per min. This reduction lagged behind the reduction in the action of nicotine, since the rate was 86 when the nicotine effect was least, and was 75 when the nicotine effect was well on the way to recovery. However, the atrial rate rose again to 98 when the nicotine effect was fully recovered.

In view of the blocking action of reserpine, experiments were made to see if reserpine would reduce the action of noradrenaline. It was found that it did so. Thus, having observed that the maximum increase in atrial rate caused by a concentration of 0.5 μg/ml. noradrenaline was 97 and 114 beats per min in two successive trials, reserpine was added to the bath in a concentration of 4 μg/ml. for a period of 68 min and then for a further period of 58 min. After the bath fluid was changed the maximum increase caused by noradrenaline was 65 beats per min. We added no more reserpine, and successive tests of noradrenaline gave a maximum increase of 86, 90, and 105 beats per min.

In another experiment it was observed that not only did reserpine reduce the maximum effect following noradrenaline, but also the duration of the effect. This finding is illustrated in Fig. 2, where each effect of noradrenaline is shown during 25 min. Curve 1 shows the response after the atria had been exposed to reserpine (4 μg/ml.) for 80 min. Curves 2, 3 and 4, obtained at intervals of 30 min, show the response to successive additions of noradrenaline as the reserpine was removed by repeated washings. The difference between curve 1 and curve 4 is very large.

Finally, we tested the effect of reserpine on the action of tyramine. Reserpine blocked the action of tyramine, and the block appeared to persist longer than the block of the action of nicotine. Thus, in one experiment, tyramine (3 μg/ml.) increased the atrial rate by 74 and 88 beats in two trials. Reserpine (4 μg/ml.)

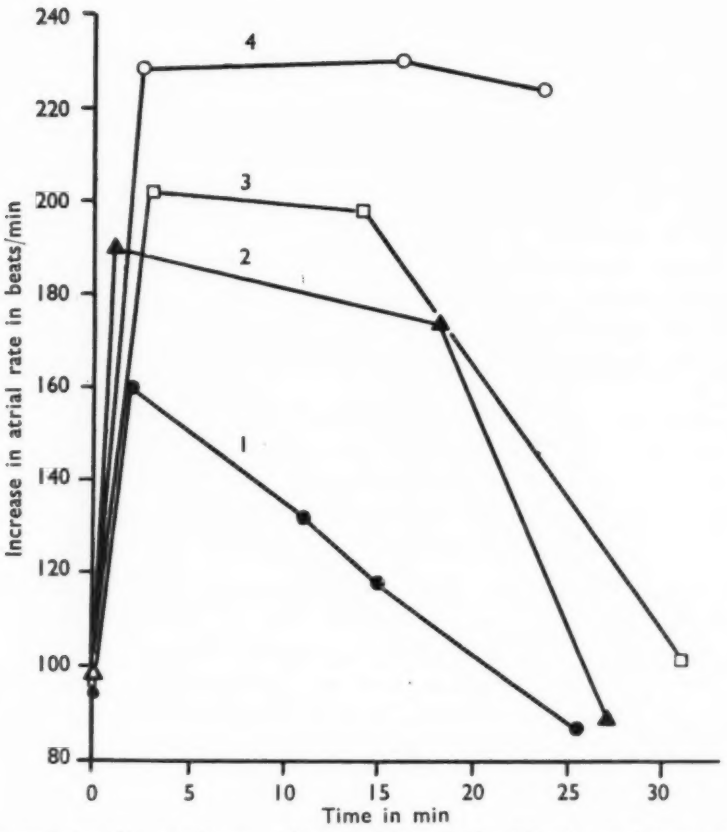

Fig. 2. After reserpine (4 μg/ml.) was added to the bath for 80 min, and then washed out, the effect of noradrenaline (0.5 μg/ml.) was small as shown in curve 1. The effect of this concentration was determined at intervals of 30 min, and curves 2, 3, and 4 were thus obtained.

was then added to the bath for 93 min and was washed out. Four successive trials of tyramine then caused no increase in rate, the fifth trial increased the rate by 14 beats, but the next four trials increased the rate only by 6, 10, 6 and 2 beats. Thus the reserpine blocked the action of tyramine for 4 hr at least.

Action of bretylium. Bretylium is a substance which blocks the effect of stimulating the postganglionic sympathetic fibres without affecting the action of noradrenaline. Its properties have been described by Boura & Green (1959). We tested the effect of bretylium on the actions of nicotine and tyramine, and observed that, while it blocked the action of nicotine, it increased the action of tyramine. In one experiment bretylium was added to the bath in a concentration of 3 μg/ml. Nicotine was tested in a concentration of 9 μg/ml., and, in two trials before the addition of bretylium, nicotine increased the atrial rate by 87 and 114 beats respectively. After bretylium had been present for 12 min nicotine increased the rate by 58 beats, and after it had been present for 56 min nicotine increased the rate by 4 beats. Tyramine, on the other hand, in a concentration of 20 μg/ml. increased the rate in the presence of bretylium by 116 beats, whereas before bretylium was added it increased the rate only by 68 beats. Bretylium thus had opposite effects on the actions of nicotine and tyramine.

DISCUSSION

From the evidence of different workers quoted in the introduction it appears that tissues such as the heart, spleen, kidney and uterus, which have a sympathetic innervation, can take up noradrenaline from the blood. In the second place, Schümann & Weigmann (1960) have shown that tyramine increases the rate of release of noradrenaline from chromaffin granules obtained from the splenic nerve by differential centrifugation.

In spite of this evidence, Kuschinsky, Lindmar, Lüllmann & Muscholl (1960) consider that tyramine does not act by liberating noradrenaline, but that, in order that tyramine may act, a certain quantity of noradrenaline must be *present*. Their evidence is based first on the finding of Muscholl (1960) that the heart of a rat treated with reserpine does not take up noradrenaline from the blood, and, second, on the ability of very low concentrations of noradrenaline to restore the action of tyramine on the isolated atria of a reserpine-treated rat. Moreover, they were able to show that the effect of the noradrenaline lasted only so long as it was present in the bath. A similar view has been expressed by Nasmyth (1960). He found that tyramine caused an increase in the amplitude of contraction of the isolated guinea-pig heart, but that this increase was no longer to be seen after several injections of tyramine had been given. At this point there was no decline in the concentration of noradrenaline in the heart. The effect of tyramine could be restored by an injection of noradrenaline. In addition Nasmyth found that perfusion of isolated hearts with Krebs solution containing tyramine for 30 min did not diminish the amount of noradrenaline present. Nasmyth concluded from this and other evidence that the potentiation of the effect of tyramine by noradrenaline occurred at a time when the noradrenaline was in the extracellular fluid.

The experiments described in this paper showed that, when atria were beating in a bath, the addition of noradrenaline to the bath increased the effect of both tyramine and nicotine on the atrial rate. This increased action was observed after the noradrenaline had been removed from the bath and the atrial rate had returned to the rate before noradrenaline was added. But the effect was not uniform. Often the first addition of noradrenaline had very little effect on the action of tyramine or of nicotine, while later additions had a great effect. Furthermore, to maintain this great effect it was usually necessary to add noradrenaline to the bath between each trial of tyramine or nicotine. But again this was not always so, and in some experiments the great effect was maintained, without further addition of noradrenaline, for as long as 3 hr. These observations are difficult to harmonize with the view that noradrenaline by its presence can so greatly potentiate the action of tyramine.

The idea that the potentiating action of noradrenaline depends on its presence in the extracellular fluid does not account for the potentiating action of noradrenaline on sympathetic stimulation which runs parallel with the effect on tyramine. Nor is it easy to conceive how the potentiating action of dopamine, of L-dopa, of *m*-tyrosine and of phenylalanine on tyramine can be explained as being due to an extracellular accumulation of noradrenaline (Burn & Rand, 1960b).

The experiments described in this paper also show that reserpine does more than displace noradrenaline from tissues where it is stored. After the atria had

been in contact with reserpine, the action of noradrenaline in increasing the atrial rate was greatly reduced, and many changes of the bath fluid were required before the reduction was no longer evident. From the experiments of Muscholl it appears that not only effector sites on which noradrenaline acts are blocked by reserpine, but storage sites also, for he found that noradrenaline was not taken up by the heart of an animal treated with reserpine (though this result was in conflict with those of Pennefather & Rand, 1960).

The block of the action of noradrenaline by reserpine is the likely explanation of various discrepancies between different observers on the effect of noradrenaline in tissues from reserpine-treated animals. For example, Macmillan (1959) observed that atria from reserpine-treated rabbits were more sensitive to the effect of noradrenaline on the rate than those from normal rabbits. Similarly, Leusen & Verbeke (1960) found that the sensitivity of the papillary muscle of the cat heart to the inotropic action of noradrenaline was regularly increased after reserpine pretreatment. On the other hand, Kuschinsky *et al.* (1960) failed to observe any increase in sensitivity to noradrenaline of the atria of the rat heart after reserpine pretreatment, no matter whether rate or force of contraction were observed. This failure may be explained by the persistence of the reserpine attachment to the receptors of the rat heart.

There are similar discrepancies for the nictitating membrane. Thus Fleming & Trendelenburg (1960) failed to observe that the nictitating membrane of the spinal cat previously treated with reserpine was supersensitive to the action of noradrenaline, but Schmitt & Schmitt (1960) recorded that supersensitivity was present in the cat under pentobarbitone anaesthesia.

There are now several examples of supersensitivity of organs to noradrenaline resulting from the administration of reserpine. It is certainly possible that, when this supersensitivity has not been observed, in some cases the supersensitivity has been masked by the blocking action of reserpine.

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UPTAKE OF LABELLED NORADRENALINE BY ISOLATED ATRIA

BY

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The uptake of labelled noradrenaline by isolated rabbit atria has been studied, and the rate of outflow of radioactivity after a period of loading has been recorded. There is first a rapid outflow presumably from extracellular space, followed by a slow outflow presumably from intracellular space. Cocaine greatly diminished the intracellular uptake. Tyramine caused an increased outflow of radioactivity from intracellular sites which was not due to the increase in atrial rate, since noradrenaline which increased the rate more than tyramine had less effect on the outflow.

In order to study the uptake of noradrenaline by isolated rabbit atria, observations have been made using noradrenaline containing ^{14}C, which was sent to us by Dr. McChesney Goodall. The atria were prepared from rabbits of 1.5 to 2.0 kg body weight as described in the preceding paper by Azarnoff and Burn (1961). When the atria were set up in the bath for about 1 hr and were observed to be contracting normally, the labelled noradrenaline was added to the fluid in the bath and was allowed to act for about 75 min. The bath was then emptied and the fluid was replaced and the rate of loss of radioactivity from the atria was determined. The effect of cocaine, of tyramine and of noradrenaline on this rate of outflow of radioactivity was observed.

METHODS

The labelled noradrenaline was in solution in sealed glass ampoules and was (±)-[2-^{14}C] noradrenaline, the radioactivity being 9.5 μC per ml. The concentration of DL-noradrenaline was 80.3 μg per ml.

In the various experiments the amounts of noradrenaline solution added to the 5 ml. bath varied from 0.1 to 0.3 ml. The noradrenaline was allowed to act for 75 min, after which time the bath was emptied. Five ml. of solution without noradrenaline was put in the bath and removed after 5 min. This procedure of emptying and filling the bath was repeated at varying intervals for 3 to 4 hr. All fluid removed from the bath was assayed for radioactivity by drying down a small aliquot on a planchette and counting in a windowless counter. No correction was made for self-absorption, the aliquot always having the same volume. At least 1,000 counts were recorded.

RESULTS

Course of loss of radioactivity. The results in one experiment (Table 2, Expt. 13) were as follows. When the atria were set up in the bath at 32° C, they were beating

at a rate of 105 per min. 0.3 ml. of labelled noradrenaline was added and after 6 min the atria were beating at a rate of 174 per min. After 75 min, represented in Table 1 as zero time, the radioactive solution was removed and the bath was filled with fresh solution without noradrenaline. The radioactivity in the solution removed was measured ; it gave 1,064,800 counts. Five minutes later the solution was again removed from the bath and replaced. The count of the solution removed was 78,055 ; as this was collected during a 5 min period the counts lost per minute were 15,611. This and the later results are given in Table 1.

TABLE 1

ESTIMATES OF RADIOACTIVITY IN BATH FLUID AFTER EXPOSING ATRIA TO LABELLED NORADRENALINE

Results were obtained from an experiment in which atria were exposed for 75 min

Time of removal of bath fluid (min)	Total count in bath fluid	Counts lost per min	Amount of radioactivity remaining in the atria
0	1,064,800	—	220,877
5	78,055	15,611	142,822
15	21,074	2,107	121,748
25	11,911	1,191	109,837
45	14,174	708	95,663
65	10,486	524	85,177
95	10,441	348	74,736
125	7,500	250	67,236
155	5,463	182	61,773
185	5,226	174	56,547
215	3,812	127	52,735

At the end of the experiment the atria were ground in a mortar with a little sand in 80% acetone until the tissue was disintegrated. The contents of the mortar and washings were made up to 10 ml., and the radioactivity present was determined. It was 52,735 counts.

The first figure in Table 1 for the Total Count in the bath fluid represented the amount of radioactivity not taken up by the atria. When the subsequent figures for Total Count in the bath fluid, which represented radioactivity leaving the atria during the period of 215 min, were added together, and to the sum of these figures (168,142) the amount still present in the atria (52,735) was added, a figure was obtained for the total uptake of radioactivity (220,877). Furthermore, this figure, when added to that for the radioactivity in the bath fluid removed at zero time (1,064,800), gave the total radioactivity originally put in the bath. This was 1,285,677. From this it was calculated that the percentage of radioactivity offered to the atria which was actually taken up was 17.2. Some of this was extracellular and some was evidently intracellular. It was possible to estimate the intracellular radioactivity by assuming that when 65 min had passed from zero time, all radioactivity which was extracellular had been removed, and that what came out of the atria after that time together with what was present at the end must have been intracellular. The sum of these counts was 85,177. This was 6.6% of the total radioactivity offered.

Another method of estimating the intracellular radioactivity was by calculating the amount of radioactivity which remained in the atria at any given time. These

figures are set out in the last column of Table 1. For example, the last figure but one in this column (56,547) was the amount of radioactivity remaining in the atria at 185 min ; it was the sum of the counts in the atria at 215 min, namely, 52,735, and the Total Count in the bath fluid at 215 min, which was 3,812.

The results in the last column of Table 1 are plotted in Fig. 1 as the amount of radioactivity remaining in the atria (on a logarithmic scale) against time on a linear scale. In Fig. 1 over the period 2 hr 35 min to 3 hr 35 min the points lay substantially

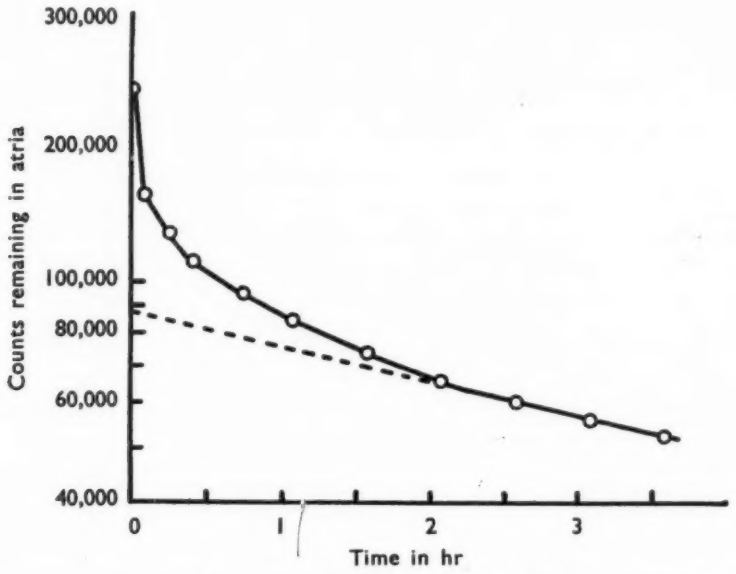

Fig. 1. Ordinates: counts per min on logarithmic scale. Abscissae: time in hr. The curve shows the rate of outflow of radioactivity from rabbit atria. The points on the right of the curve from 2 hr 35 min to 3 hr 35 min were practically linear, and, when extrapolated to the left, cut the ordinate at 88,000 counts.

on a straight line which was extrapolated back to zero time. It cut the ordinate at 88,000 counts. When this figure was expressed as a percentage of the total offered, a second estimate of the intracellular radioactivity was obtained which was 6.8%. This agreed with the first estimate of 6.6%.

When 6.8% was taken as being the proportion of radioactivity offered which became intracellular, then since the total radioactivity taken up was 17.2% the radioactivity which remained extracellular must have been 10.4%. In Table 2 the figures for the uptake which was extracellular and for the uptake which became intracellular are given for 13 experiments.

Of the 13 experiments, 9 were carried out in Oxford and 4 in St. Louis. If 3 experiments in which cocaine was present are excluded, Table 2 shows that the total uptake in the Oxford experiments (both " extracellular " and " intracellular ") varied from 24.9% to 37.8% of the total radioactivity offered, the mean figure being 31.4%. The total uptake in the St. Louis experiments was less, being only 17.5%. There was, however, no difference between the extracellular and intracellular proportions in the two places. In Oxford the rabbits were vigorous brown

TABLE 2

PERCENTAGE OF EXTRACELLULAR AND INTRACELLULAR UPTAKE OF RADIOACTIVITY

Laboratory	Expt no.	Period of uptake min	Uptake as % of radioactivity offered		Drug
			Extracellular	Intracellular	
Oxford	1	87	18·9	18·9	
	2	65	11·6	13·3	
	3	70	13·1	16·1	
	4	84	14·0	20·0	
	5	67	14·5	13·3	
	6	63	17·6	13·2	
	7	68	18·4	15·1	
	8	68	13·7	6·4	Cocaine 10^{-5} g/ml.
	9	96	15·7	17·7	
St. Louis	10	87	9·7	8·1	
	11	62	9·9	3·4	Cocaine 10^{-5} g/ml.
	12	75	8·1	2·6	Cocaine 10^{-5} g/ml.
	13	75	10·4	6·8	

rabbits, while in St. Louis they were less vigorous albino rabbits, though of similar weight.

Three experiments were carried out in the presence of cocaine hydrochloride 10^{-5} g/ml. This was added to the bath before the labelled noradrenaline was put in, and it was freshly added at each change of bath fluid throughout the experiment.

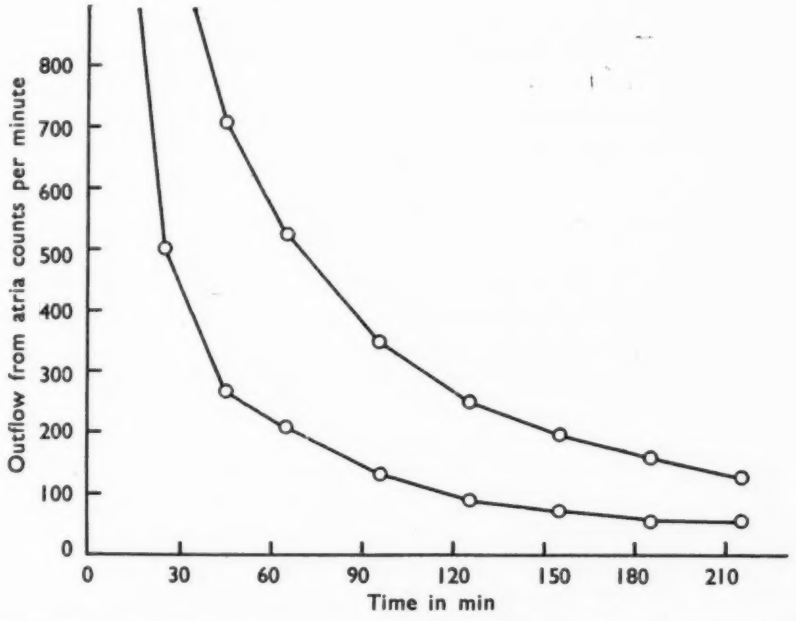

Fig. 2. The outflow of radioactivity from the atria in counts per min (ordinates) during 215 min on removal of radioactive noradrenaline from the bath. The upper curve was obtained in the absence of cocaine. The lower curve was obtained in the presence of cocaine hydrochloride 10^{-5} g/ml. There was less radioactive noradrenaline to leave the atria from intracellular sites.

The results showed that in the presence of cocaine the uptake of labelled noradrenaline which became intracellular was greatly reduced.

In the one experiment with cocaine in Oxford the "intracellular" uptake was 6.4% as compared with a mean figure of 15.9% for experiments without cocaine, the lowest value being 13.2%. In the two experiments with cocaine in St. Louis, the "intracellular" uptake was 3.0% as compared with 7.5% for two experiments without cocaine. The effect of cocaine is shown in Fig. 2. Thus cocaine 10^{-5} g/ml. reduced the "intracellular" uptake to 40% of the uptake in the absence of cocaine both in Oxford and in St. Louis.

Effect of cocaine on outflow of radioactivity. In the experiments in which cocaine was present while the radioactivity was being taken up, cocaine was also present in the same concentration while the radioactivity was flowing out. It was unlikely that cocaine would influence the rate at which radioactivity left the extracellular

TABLE 3

CALCULATED RATE OF LOSS OF RADIOACTIVITY IN PRESENCE AND ABSENCE OF COCAINE

Laboratory	Cocaine 10^{-5} g/ml.	Velocities, hr^{-1}	Mean velocity hr^{-1}
Oxford	Absent	0·12, 0·05, 0·073, 0·075, 0·144, 0·138, 0·144	0·106
	Present	0·157	0·157
St. Louis	Absent	0·111, 0·160	0·135
	Present	0·075, 0·125	0·100

space, but it might influence the rate at which it left the intracellular space. Table 3 gives figures for the velocity at which the "intracellular" radioactivity left the atria when cocaine was present and when cocaine was absent. The velocity constant was derived from the expression

$$\frac{1}{t}\log_n \frac{a}{a-x}$$

where t was the time in hr, a was the initial value of the "intracellular" uptake (determined by extrapolation as described above), and $(a - x)$ was the value at time t. Table 3 shows that there was no consistent difference between the velocities at Oxford and at St. Louis on the one hand, nor between the velocities in the presence and absence of cocaine on the other.

Effect of tyramine and noradrenaline on outflow of radioactivity. The effect of tyramine on the outflow of radioactivity from the atria was studied by adding tyramine to the bath 60 min after zero time, when there was presumably no extracellular radioactivity remaining. The result in Expt. 5 is shown in Fig. 3. The concentration of tyramine hydrochloride was 16 μg/ml., which was allowed to act for 10 min. The bath was emptied, and refilled with the modified Ringer solution (see preceding paper) to which the same concentration of tyramine was again added for a further 10 min. The outflow was then studied with modified

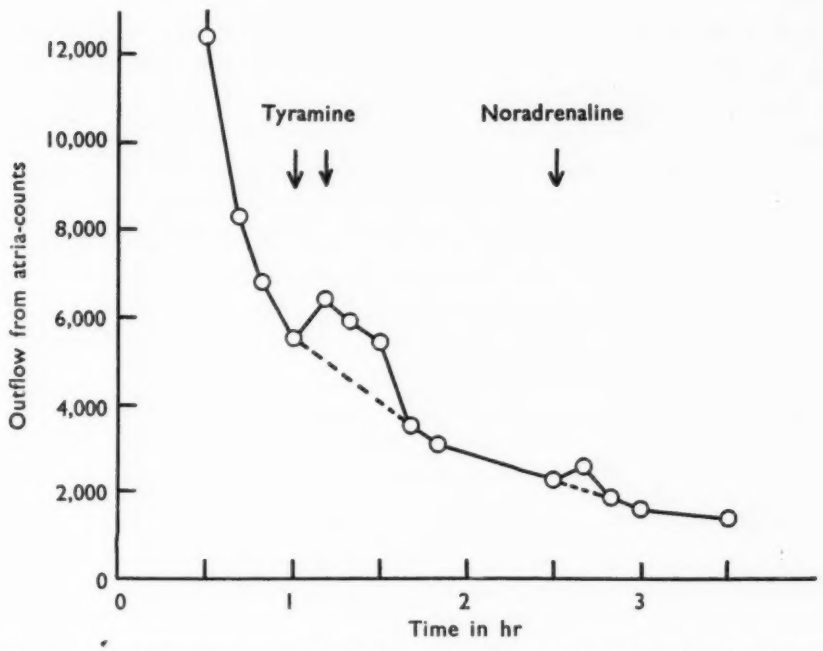

Fig. 3. The action of tyramine in raising the outflow of radioactivity, and a similar but smaller action of noradrenaline.

Ringer solution in the bath until 2.5 hr (from zero time) had elapsed, when 8 μg noradrenaline was added and allowed to act for 10 minutes. The increase in outflow of radioactivity was measured by estimating the outflow that would have been expected if tyramine (or noradrenaline) had not been added. The estimates are shown in Fig. 3 by the dotted lines. When tyramine was added the actual outflow over the 30 min period during which the outflow increased was 17,700 counts, whereas the estimated outflow was 13,500 counts. Thus tyramine caused an increase of 31%.

TABLE 4

EFFECT OF TYRAMINE AND NORADRENALINE ON OUTFLOW OF RADIOACTIVITY

Expt.	Drug	Concentration in bath μg/ml.	Time of action in min	Increase of atrial rate beats/min	Increase in outflow of radioactivity
3	Tyramine	4	20	68	35
4	Tyramine	16	20	77	31
5	Tyramine	16	20	64	31
6	Tyramine	32	10	48	24
7	Tyramine	16	10	50	52
5	Noradrenaline	8	10	86	24
6	Noradrenaline	5	10	80	8
7	Noradrenaline	2·5	10	80	20

In experiments 5, 6 and 7, tyramine and noradrenaline were compared; in each experiment tyramine caused a greater increase of the outflow radioactivity but a smaller effect on the atrial rate (Table 4). In experiments 6 and 7, both substances

were added to the bath for the same time, and their effects were strictly comparable ; tyramine caused a mean increase of 38% in outflow, whereas noradrenaline caused a mean increase of 14%. However, tyramine caused a mean increase in atrial rate of 49 beats per min, whereas noradrenaline caused a mean increase of 80 beats per min. The increase in outflow represented a very small fraction of the radioactivity remaining in the atria, for in Expt. 7, in which tyramine caused an increase of 52%, the increase represented only 1.2% of the total radioactivity in the atria.

DISCUSSION

The observations showed that when rabbit atria were suspended in a bath, to which radioactive noradrenaline was added, they took up radioactivity. Muscholl (1960) has shown that guinea-pig atria took up non-radioactive noradrenaline in similar circumstances, and that the atrial content of noradrenaline estimated biologically was doubled. When the atria in our experiments were placed in a non-radioactive solution, there was a rapid outflow of radioactivity during the first 25 min, probably coming from noradrenaline which was extracellular. Then after 65 min from zero time there was a much slower outflow which appeared to come from noradrenaline which was intracellular.

The evidence for the slow outflow being an outflow of noradrenaline which was intracellular was strengthened by the action of cocaine. Trendelenburg (1959) showed that the rate of disappearance of noradrenaline from the blood of a spinal cat was much slower in the presence of cocaine. The work of Burn & Rand (1958) led them to suggest that the disappearance of noradrenaline from the blood was due to uptake by tissues with a sympathetic innervation, and Macmillan (1959) suggested that cocaine prevented the uptake of noradrenaline by the tissues. Whitby, Hertting & Axelrod (1960), using tritiated noradrenaline, measured the uptake of noradrenaline in the anaesthetized cat by the heart, spleen and adrenal glands, and found that the uptake was greatly reduced in the presence of cocaine. When cocaine was added to the bath fluid in the experiments described in this paper, the slow outflow was much smaller. Thus, in the experiments shown in Fig. 2, the outflow at 120 min after zero time was 90 counts per min in the presence of cocaine, but it was 250 counts per min in the absence of cocaine, suggesting that in the absence of cocaine there was a much larger intracellular store of radioactive noradrenaline.

The addition of tyramine to the bath after 1 hr from zero time caused a rise in outflow of radioactivity in five experiments. The mean rise was 35%. This rise might have been due to the increase in the rate of atrial contractions caused by the tyramine. However, when noradrenaline (not radioactive) was added to the bath, producing a much greater rise in the atrial rate, the rise in outflow of radioactivity was much smaller. The conclusion thus follows that the rise in outflow of radioactivity caused by tyramine was not due to the increase in rate, and could therefore be attributed to an increased discharge of radioactive noradrenaline from its intracellular position. Thus this increased output of radioactive noradrenaline following the addition of tyramine to the bath provided further evidence for the view that tyramine acts by liberating noradrenaline from an intracellular site.

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EFFECT OF COCAINE AND RELATED DRUGS ON THE UPTAKE OF NORADRENALINE BY HEART AND SPLEEN

BY

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Noradrenaline uptake by heart and spleen after intravenous infusion of noradrenaline was measured in the pithed rat. Cocaine, given before the infusion, inhibited the noradrenaline uptake in relation (*a*) to the dose administered and (*b*) to the amount of noradrenaline infused. There was an association between increase in the pressor response to a test dose of noradrenaline and inhibition of the uptake by the heart. Drugs related chemically to cocaine, such as α-cocaine, amethocaine, and atropine, did not alter the noradrenaline uptake or potentiate the blood pressure response to noradrenaline. The noradrenaline uptake by the heart was unchanged after dibenamine, but blocked by the dichloro-analogue of isoprenaline. It was concluded that cocaine specifically prevented the uptake of noradrenaline by tissues, thus increasing the amount of noradrenaline available for combination with adrenergic receptors. The dichloro-analogue of isoprenaline appeared to block both uptake by the heart and the combination with receptors.

Since Fröhlich & Loewi (1910) first described sensitization by cocaine of various organs to adrenaline, different explanations for the potentiating effect of cocaine on responses to catechol amines have been offered. Recently Trendelenburg (1959) reinvestigated the question whether cocaine causes supersensitivity by preventing the destruction of injected noradrenaline. He found that cocaine delayed the inactivation of noradrenaline, and that the elevated plasma concentrations of noradrenaline fully accounted for the increase in blood pressure responses. Trendelenburg (1959) suggested that inactivation may be caused, amongst other means, by a binding process, and such binding by many tissues was demonstrated by Axelrod, Weil-Malherbe & Tomchick (1959) to inactivate circulating adrenaline.

From indirect evidence, Macmillan (1959) concluded that cocaine prevented the uptake of catechol amines by tissue stores, thereby increasing the amount of noradrenaline acting on sympathetic receptors. In a direct test of this hypothesis, Muscholl (1960a) showed that a noradrenaline infusion given to a rat approximately doubled the concentration of noradrenaline in heart and spleen, and that cocaine blocked this uptake. Blocking by cocaine of the accumulation of tritium-labelled noradrenaline in various organs has also been observed by Whitby, Hertting & Axelrod (1960).

The purpose of the present investigation was to investigate the specificity of the effect of cocaine on the uptake of noradrenaline and the quantitative relation between this uptake and the potentiation of the effects of noradrenaline.

METHODS

Male or female rats, weighing 170 to 250 g, were anaesthetized with ether, pithed and maintained by artificial respiration (Muscholl & Vogt, 1957). The blood pressure was recorded from the carotid artery with a Condon-type mercury manometer. A femoral vein was cannulated for injection of drugs. Infusions of noradrenaline dissolved in saline were given into this cannula at a constant rate for a period of 20 min using a motor-driven syringe. After infusions or injections the cannula was rinsed through with saline. The total volume of saline administered in any one experiment never exceeded 3 ml.

Five or twenty min after the end of the infusion of noradrenaline the heart was excised. Atria and large vessels were removed, the myocardium dried on filter paper and weighed. The tissue was homogenized, extracted in acid ethanol, the extract purified and chromatographed on paper. The eluates of the regions containing the noradrenaline and adrenaline were assayed individually on the blood pressure of the pithed rat and on the isolated atropinized rat uterus stimulated with oxytocin, as described by Muscholl (1959).

In some experiments the concentration of noradrenaline in the spleen was estimated by preparing extracts as described for cat heart (Muscholl, 1959).

The following substances were used: (−)-Noradrenaline base dissolved in hydrochloric acid; (−)-adrenaline-(+)-bitartrate (doses and concentrations refer to the bases). Cocaine hydrochloride; amethocaine (Tetracaine) hydrochloride; atropine sulphate; α-cocaine hydrochloride; dibenamine (N,N-dibenzyl-β-chloroethylamine hydrochloride); the dichloro-analogue of isoprenaline, 1-(3′,4′-dichlorophenyl)-2-isopropylaminoethanol hydrochloride (doses refer to the salts).

RESULTS

Action of cocaine on the uptake of noradrenaline by heart and spleen. The concentration of noradrenaline in the heart of 9 normal rats was found to be 0.59 ± 0.02 μg/g (mean ± s.e. of the mean). This agrees well with the value of 0.61 μg/g reported previously by Muscholl (1959). Likewise, the concentration of adrenaline in the normal hearts (46 ± 5 ng/g) fell within the range found by Muscholl (1959).

TABLE 1

CONTENT OF NORADRENALINE AND ADRENALINE IN THE HEART AND OF NORADRENALINE IN THE SPLEEN OF THE PITHED RAT AFTER INFUSION

Concentrations in μg or ng/g fresh tissue, mean ± s.e. of the mean. Number of estimations in brackets. * Individual figures. Rats killed 5 min after cessation of intravenous infusion of saline or 30 min after intravenous administration of the drug. Dichloroisoprenaline was injected into tail vein

			Heart		Spleen
Treatment	Drug	Dose mg/kg	Noradrenaline μg/g	Adrenaline ng/g	Noradrenaline μg/g
—	Untreated controls	—	0·59 ± 0·02 (9)	46 ± 5 (5)	0·19 ± 0·04 (6)
Pithed	Saline infusion, 3 ml./20 min	—	0·58 ± 0·04 (4)	38 ± 10 (4)	0·19 ± 0·07 (3)
Pithed	Cocaine	10	0·61*; 0·60 (2)	40; 21 (2)	0·09; 0·17 (2)
Pithed	Dibenamine	25–50	0·55 ± 0·02 (3)	—	—
—	Dichloroisoprenaline	30	0·60 ± 0·01 (3)	—	—

The concentration of noradrenaline in the spleen and the concentrations of the catechol amines in the heart were not altered by the pithing procedure used (Table 1). Control values given in Table 2 represent the means of untreated rats and of pithed control animals infused with saline.

In hearts excised 5 min after the end of an infusion of 20 μg of noradrenaline administered during a period of 20 min, the concentration of noradrenaline was 1.05 ± 0.04 μg/g. This corresponded to a net uptake of 0.46 μg/g or 78% of the control value. The adrenaline concentration of the heart was not affected by the noradrenaline infusion (Table 2), but the noradrenaline concentration of the spleen had risen by 0.15 μg/g (79% increase).

TABLE 2

EFFECT OF COCAINE ON THE NORADRENALINE UPTAKE BY THE HEART AND BY THE SPLEEN AFTER AN INFUSION OF NORADRENALINE

Concentrations in μg or ng/g fresh tissue, mean $\pm$ s.e. of the mean. Number of estimations in brackets. Organs were removed 5 min after end of infusion

Dose of cocaine mg/kg	μg of noradrenaline infused in 20 min	Heart		Spleen
		Noradrenaline μg/g	Adrenaline ng/g	Noradrenaline μg/g
—	—	0.59 ± 0.02 (13)	42 ± 5 (9)	0.19 ± 0.03 (9)
—	20	1.05 ± 0.04 (21)	36 ± 9 (10)	0.34 ± 0.02 (10)
10	20	0.85 ± 0.03 (4)	32 ± 5 (4)	0.26 ± 0.04 (4)
20	20	0.74 ± 0.08 (8)	35 ± 8 (6)	0.22 ± 0.02 (8)
—	5	0.85 ± 0.03 (10)	—	—
2·5	5	0.62 ± 0.03 (5)	—	—

In experiments in which the heart was excised 20 min after the infusion terminated, the concentration of noradrenaline was still high, being 1.06 ± 0.08 μg/g ($n = 5$).

A dose of 10 or 20 mg/kg of cocaine given 5 to 15 min before the noradrenaline infusion reduced significantly the net uptake of noradrenaline by heart and spleen measured 5 min after the end of the infusion (Table 2). Again, the adrenaline

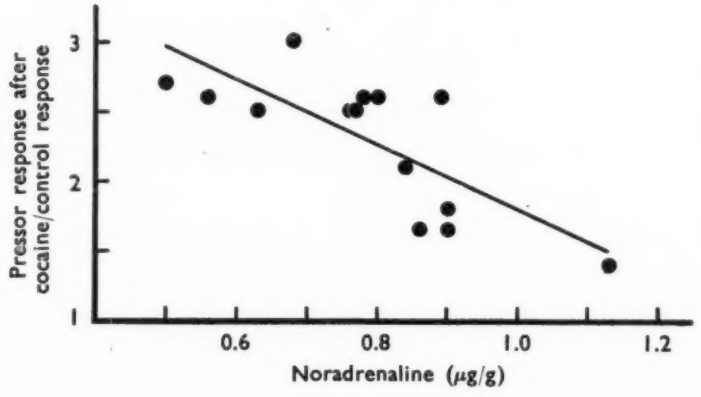

Fig. 1. Potentiation of the pressor response to noradrenaline by cocaine plotted against the noradrenaline concentration in the rat heart after a noradrenaline infusion. Each point represents an estimation on a pithed rat injected with cocaine (10 to 20 mg/kg) and given a noradrenaline infusion of 20 μg in 20 min. Ordinate: Ratio of the pressor response to a test dose of noradrenaline (2 to 5 ng) after cocaine/control response. Abscissa: Noradrenaline concentration of the heart measured 5 to 20 min after terminating infusion. The calculated regression is significant at the 1% level.

concentration of the heart was unchanged. Concentrations of noradrenaline measured 20 min after terminating the infusion (0.74 ± 0.05 μg/g, n = 6) were also lowered by the previous injection of 10 mg/kg of cocaine.

In 14 out of 18 experiments the pressor response to a small test dose of noradrenaline was recorded before and after cocaine (10 to 20 mg/kg) had been injected. Subsequently, 20 μg of noradrenaline was infused. The pressor response to noradrenaline after cocaine varied inversely to its concentration measured in the heart after the infusion, and this relation could be expressed as a regression (Fig. 1). Since increases of concentrations of noradrenaline in the hearts above control values (0.59 μg/g) gave an estimate of the uptake by the heart, the results in Fig. 1 show that, with increasing depression of the uptake of noradrenaline by cocaine, there was increasing sensitization of the pressor response to noradrenaline.

Control experiments were made to eliminate the possibility that cocaine acted by reducing the concentration of noradrenaline in the heart *per se* rather than by preventing the uptake of exogenous noradrenaline. Table 1 shows that cocaine did not alter the concentrations of catechol amines in heart and spleen significantly. In two other experiments the amount of noradrenaline accumulated by the heart during a noradrenaline infusion of 20 μg did not decrease after the subsequent injection of 10 or 20 mg/kg of cocaine; the concentrations of noradrenaline in the hearts were 1.04 and 1.05 μg/g, respectively, and thus did not differ from the mean of 1.05 μg/g for rats receiving a noradrenaline infusion without pretreatment with cocaine.

Injection of a small dose (2.5 mg/kg) of cocaine had no effect on the uptake of noradrenaline by the heart if 20 μg of noradrenaline was infused, but completely blocked the uptake of noradrenaline when only 5 μg was infused (Table 2). The results of experiments using doses of 0.15 to 2.5 mg/kg of cocaine and infusions

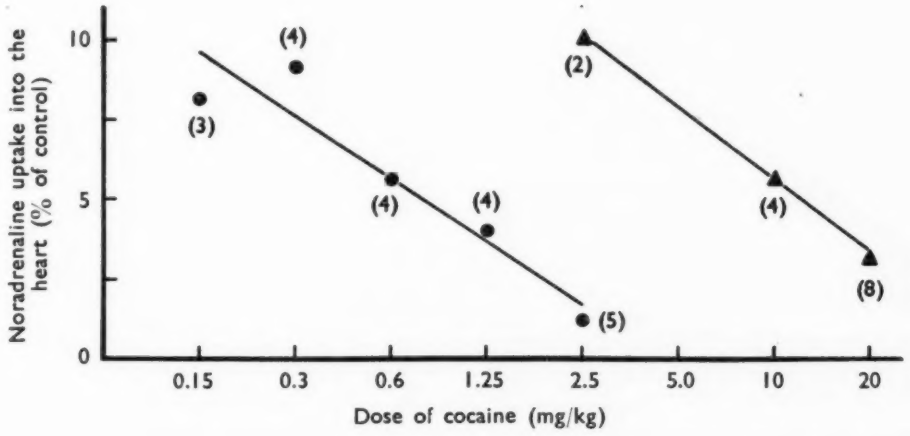

Fig. 2. Dose of cocaine plotted against % inhibition of noradrenaline uptake by the heart. Hearts removed 5 min after terminating noradrenaline infusion of 5 μg (●) or 20 μg (▲) per rat given in 20 min. Number of estimations in brackets. Noradrenaline uptake calculated as % of control values from Table 2 (for 5 μg infusions 0% = 0.59 and 100% = 0.85 μg/g; for 20 μg infusions 0% = 0.59 and 100% = 1.05 μg/g).

of 5 μg of noradrenaline are summarized in Fig. 2. It can be calculated from the regression lines that 0.8 mg/kg of cocaine caused 50% inhibition of uptake when 5 μg noradrenaline was infused, whereas 11.6 mg/kg of cocaine was required to produce the same percentage inhibition with infusion of 20 μg of noradrenaline.

Since there was little variation in the heart weights between the experimental groups, changes in concentrations also represented changes in total content of catechol amines.

Drugs chemically related to cocaine. These effects of cocaine might be related to its local anaesthetic action. Amethocaine, which has about 4 times the local anaesthetic activity of cocaine, did not increase the blood pressure response to noradrenaline in the pithed rat, nor did it inhibit the uptake of noradrenaline by the heart (Table 3).

Atropine, which is structurally related to cocaine, had no sensitizing effect on the noradrenaline pressor response and neither did it alter the uptake of noradrenaline (Table 3).

TABLE 3

EFFECT OF DRUGS ON THE NORADRENALINE UPTAKE INTO THE HEART AFTER AN INFUSION OF NORADRENALINE

Mean of noradrenaline concentration in μg/g$\pm$s.e. of the mean. Number of estimations in brackets. Hearts were removed 5 min after cessation of infusion of 20 μg of noradrenaline in 20 min

Drug given before noradrenaline infusion	mg/kg	Heart noradrenaline μg/g
No drug	—	1·05$\pm$0·04 (21)
Amethocaine	5–10	1·24$\pm$0·10 (3)
Atropine	20	1·02$\pm$0·04 (3)
α-Cocaine	20	1·04$\pm$0·05 (5)
Dibenamine	25–50	0·91$\pm$0·10 (5)
Dichloroisoprenaline	30	0·64$\pm$0·05 (5)

Foster, Ing & Varagić (1955) have shown that α-cocaine, a structural isomer of cocaine, has some local anaesthetic properties, inhibits monoamine oxidase to the same extent as cocaine but lacks the potentiating action on the catechol amines in the spinal cat. In 5 rats, α-cocaine had no action on the blood pressure response to noradrenaline or on the uptake of noradrenaline by the heart (Table 3).

Adrenaline-blocking agents. According to Brown & Gillespie (1957), blocking of the adrenaline receptors increased the noradrenaline output of the spleen, presumably by preventing the combination of noradrenaline with the receptors. In the present study two blocking agents were tested. Dibenamine (25 to 50 mg/kg), which blocks only α-receptors (Ahlquist, 1958), and the dichloro-analogue of isoprenaline (30 mg/kg), which blocks only β-receptors (Powell & Slater, 1958 ; Moran & Perkins, 1958). By themselves neither modified the concentration of noradrenaline in the heart (Table 1). In the pithed rat the maximum pressor effect of noradrenaline infused at a rate of 20 μg/20 min was 88$\pm$4 mm Hg, and it occurred within the first few minutes of starting the infusion. Dibenamine, injected before

the noradrenaline infusion, abolished completely the initial pressor effect of noradrenaline. However, during the course of the infusion the blood pressure rose steadily, reaching a maximum at the end of the infusion. Nevertheless, this rise of blood pressure (24 ± 4 mm Hg) was significantly lower than the maximum rise in control animals not given dibenamine. The uptake of noradrenaline by the heart was not significantly affected by doses of dibenamine which effectively blocked the α-receptors (Table 3).

The dichloro-analogue of isoprenaline, injected before the noradrenaline infusion, significantly increased the maximum blood pressure rise caused by noradrenaline (114 ± 4 mm Hg). The uptake of noradrenaline by the heart was completely blocked by the analogue (Table 3).

DISCUSSION

The results reported here show that cocaine blocked the noradrenaline uptake from the circulating blood by heart and spleen without affecting the endogenous concentrations of noradrenaline and adrenaline in these organs. Muscholl (1960b) found reserpine blocked the uptake of noradrenaline by the heart, but, in contrast to cocaine, it decreased the endogenous concentration of noradrenaline. There may be, therefore, a difference in the mechanisms by which cocaine and reserpine prevent the uptake by tissues of noradrenaline from the circulating blood.

The present results are in agreement with the work of Whitby *et al.* (1960), who found that an injection of 5 μg/kg of cocaine depressed the uptake of tritium-labelled noradrenaline by the adrenal glands, heart and spleen of the cat. There was a corresponding elevation of blood concentrations of noradrenaline within the first 3 min following the injection, as noted by Trendelenburg (1959).

The inhibition of the uptake of noradrenaline was related to the potentiation of the responses to noradrenaline observed after cocaine (Fig. 1). Moreover, with increasing doses of noradrenaline, much larger doses of cocaine were required to inhibit uptake (Fig. 2). Finally, the specificity of the action of cocaine was demonstrated. Of the four chemically related drugs, cocaine, atropine, amethocaine, and α-cocaine, only cocaine inhibited the noradrenaline uptake by the heart and caused supersensitivity towards noradrenaline.

Doses as small as 0.2 mg/kg of cocaine are known to potentiate the actions of noradrenaline on the nictitating membrane and spleen of the spinal cat (Trendelenburg, 1959), but, in the present experiments, doses of 0.15 and 0.3 mg/kg did not significantly reduce the uptake of noradrenaline by the heart (Fig. 2). This apparant lack of correlation between the effects of cocaine on the uptake and the potentiation of noradrenaline may be explained by the following considerations. With infusions of 20 and 5 μg of noradrenaline, the doses of cocaine needed to produce a 50% inhibition of the uptake of noradrenaline were 11.6 and 0.8 mg/kg, respectively Therefore relatively much smaller doses of cocaine are required to block the uptake of somewhat smaller amounts of infused noradrenaline. The doses of noradrenaline injected by Trendelenburg (1959) were smaller (3 to 16 μg/kg) than the lowest dose infused in the present experiments (5 μg per rat being about 25 μg/kg).

Although the action of cocaine on the noradrenaline uptake was specific in the sense that some closely related drugs did not possess such an effect, blocking of the noradrenaline uptake is also known to occur with reserpine and was seen with the dichloro-analogue of isoprenaline. The latter, like cocaine, did not affect the endogenous concentrations of noradrenaline in the heart, but the type of block exerted by these two drugs was quite dissimilar. It might be assumed that cocaine, apart from blocking the sites which take up noradrenaline from the blood, does not block the adrenaline receptors of the heart, since it does not block but potentiates noradrenaline effects on atria (Macmillan, 1959). The isoprenaline analogue, on the other hand, exerts a specific block of the adrenaline receptors of the heart (Moran & Perkins, 1958). Both agents reduce the uptake of noradrenaline by the heart to the same extent if employed in a sufficient dose. This indicates that block of adrenaline receptors by the dichloro-analogue of isoprenaline was not reflected in a greater inhibition of the noradrenaline uptake. Possibly the noradrenaline which combined with adrenergic receptors was metabolized and therefore could not be detected by the method used, whereas the noradrenaline taken up by the tissue stores was protected and could be estimated after extracting the whole heart. It is interesting to note that dibenamine, which does not block the receptors responsible for increase in rate and force of contraction of the heart (Nickerson, 1949), did not prevent noradrenaline from being held in the tissues.

From the present experiments the following conclusions may be drawn: Injected noradrenaline not only combines with adrenergic receptors but is stored in considerable concentrations at other sites. Cocaine prevents the uptake into these sites, thereby increasing the amount of noradrenaline available for combination with the adrenaline receptors. The dichloro-analogue of isoprenaline blocks *both* the uptake of noradrenaline into the stores and the combination with the adrenaline receptors.

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SOME EFFECTS OF CORTICOSTEROIDS ON THE METABOLISM OF HISTAMINE AND 5-HYDROXYTRYPTAMINE IN THE RAT

BY

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Daily intramuscular injections of cortisone, prednisolone, triamcinolone, fludrocortisone and 2-methylfludrocortisone reduced the histidine decarboxylase activity of the rat liver, but increased the enzyme activity of the pyloric stomach. Injections of histamine liberators or exposure to cold produced similar changes. After adrenalectomy, the histidine decarboxylase activities of the liver and pyloric stomach were unaltered, but the histaminase activity of the ileum was reduced. The 5-hydroxytryptophan decarboxylase activities of rat liver and kidney were not altered by treatment with corticosteroids.

The histamine and 5-hydroxytryptamine contents of rat skin and small intestine are lowered by injections of corticosteroids, whereas those of the pyloric stomach are raised (Telford & West, 1960). Analogues of cortisone which possess high glucocorticoid activity are the most active. After adrenalectomy the histamine and 5-hydroxytryptamine contents of most rat tissues are raised, especially if the rats are given water to drink instead of saline (Hicks & West, 1958b). The secretion of the adrenal cortex may therefore control the tissue reserves of these amines, as suggested by Hicks & West (1958a). The present work was undertaken to test this hypothesis further, by investigating the effect of corticosteroids, stress and adrenalectomy on histamine and 5-hydroxytryptamine metabolism.

METHODS

Groups of 6 female rats of Wistar strain weighing 120 to 180 g were used in all experiments. They were fed on a cube diet (No. 41B, Associated London Flour Millers), allowed drinking water *ad lib.* and housed at 21 ± 0.5° C.

Drug treatment

Aqueous suspensions of the corticosteroids (10 mg/kg) were injected intramuscularly every day for 4 or 9 days. The steroids used were deoxycortone acetate, cortisone acetate, prednisolone, triamcinolone, fludrocortisone and 2-methylfludrocortisone (9α-fluoro-11β, 17α, 21-trihydroxy-2α-methylpregn-4-ene-3, 20 dione). Control rats received the corresponding volumes of the suspending fluid or saline. The animals were killed 24 hr after the last injection.

Exposure to cold

Rats were placed in individual cages in a cold room at 2 ± 0.5° C for 5 hr, 15 hr, 40 hr and 64 hr. On removal from the cold room they were immediately killed.

Depletion of tissue amines

The histamine liberators, compound 48/80 and polymyxin B, were used in the dose schedules employed by Parratt & West (1957b):

(a) *Short-term treatment.* Two doses, one in the morning and one in the afternoon, given intraperitoneally to groups of rats which were killed 1, 3 and 9 days later.

(b) *Long-term treatment.* Seven doses of compound 48/80 over 4 days or 6 doses of polymyxin B over 3 days given intraperitoneally to groups of rats which were killed 1, 5 and 13 days after the last injection.

Adrenalectomy

Bilateral adrenalectomy was performed under ether anaesthesia. Thereafter the rats were given their normal diet and water to drink for 4 days before being killed. Mock-adrenalectomized rats were similarly treated.

Measurement of histamine formation

The *in vitro* method of Waton (1956), slightly modified and described in detail by Telford & West (1961a, 1961b), was used. The sources of histidine decarboxylase studied in the present work were the liver and pyloric stomach, since other tissues have been shown to possess little or no activity. Briefly, pooled tissue from 6 rats was ground in a glass mortar with a little sand and Tyrode solution (5 ml./g tissue). The resulting homogenate was allowed to stand and the supernatant fluid was removed for incubation. The composition of the incubation mixture was as follows:

Tissue homogenate (400 mg)	2.0 ml.
L-Histidine (neutralized, 15 mg/ml.)	0.5 ml.
Aminoguanidine (neutralized, 10 mg/ml.)	0.05 ml.
Benzene	20 mg
Phosphate buffer (M/20 K_2HPO_4)	2.45 ml.

For incubations of liver homogenates a buffer of *p*H 8.0 was used; for homogenates of pyloric stomach, *p*H 7.0. The mixture was immediately shaken and incubated for 3 hr at 37° C. The reaction was stopped by reducing the *p*H of the solution to 4.0 with N-hydrochloric acid and by cooling to 4° C. After neutralizing the mixture with N-sodium hydroxide, its histamine content was determined. In each experiment, mixtures without the substrate (histidine) were also incubated and assayed for histamine. The final volume of all mixtures was 5 ml. Each incubation was carried out in duplicate. The mean histamine content of the mixtures incubated in the presence of histidine less the mean histamine content of mixtures incubated in the absence of histidine gave the amount of histamine formed from histidine. The amounts of histamine (μg) formed per gramme of tissue and per tissue were used as indices of histidine decarboxylase activity; in control rats, these values ($\pm$s.e.) were 10.2 ± 1.9 μg/g and 71.4 ± 4.3 μg for liver and 15.6 ± 2.2 μg/g and 7.0 ± 1.1 μg for pyloric stomach.

Measurement of 5-hydroxytryptamine formation

The method of Gaddum & Giarman (1956), modified by West (1958), was used for the *in vitro* determination of tissue 5-hydroxytryptophan decarboxylase activity. Briefly, pooled tissue was ground in a glass mortar with a little sand and M/15 phosphate buffer (2 ml./g tissue) at *p*H 8.0. Pyridoxal phosphate (100 μg), iproniazid (100 μg) and phosphate buffer (2.8 ml.) were added to an aliquot of the homogenate containing 800 mg of tissue. Racemic 5-hydroxytryptophan (400 μg) was added last. The volume of the mixture was 5 ml. The mixture was immediately shaken and incubated for 1 hr at 37° C. The reaction was stopped by reducing the *p*H to 5.0 with N-hydrochloric acid and the 5-hydroxytryptamine content of the solution determined. The quantity of 5-hydroxytryptamine formed per gramme of tissue was used as the index of 5-hydroxytryptophan decarboxylase activity. Duplicate incubations were performed in each experiment.

Measurement of histaminase activity

The histaminase activity of homogenates of rat ileum was estimated by the method of Wicksell (1949), using a histamine concentration of 100 μg/g tissue.

Assay procedures for histamine and 5-hydroxytryptamine

Histamine was assayed on the isolated ileum of the guinea-pig and 5-hydroxytryptamine was assayed on the isolated uterus of the rat. The methods have been described in detail elsewhere (Parratt & West, 1957a). Each value of histamine and 5-hydroxytryptamine refers to the base. Statistical analysis showed that values differing from the means by more than 25% were significant ($P=0.95$).

RESULTS

The effect of corticosteroids on histamine formation

Liver. The histidine decarboxylase activity of rat liver after treatment with corticosteroids for 4 days is shown in Fig. 1. The enzyme activity was unaffected by deoxycortone and cortisone, but was reduced by the more potent glucocorticoids. When treatment was extended to 9 days, the enzyme activity was not further reduced by prednisolone, triamcinolone, fludrocortisone and 2-methylfludrocortisone, and deoxycortone remained ineffective ; cortisone, however, reduced the rate of formation of histamine.

Pyloric stomach. In this tissue, the glucocorticoids increased the rate of histamine formation (Fig. 1). As in the liver, the most active steroids were those possessing the highest glucocorticoid activity. For example, triamcinolone increased the

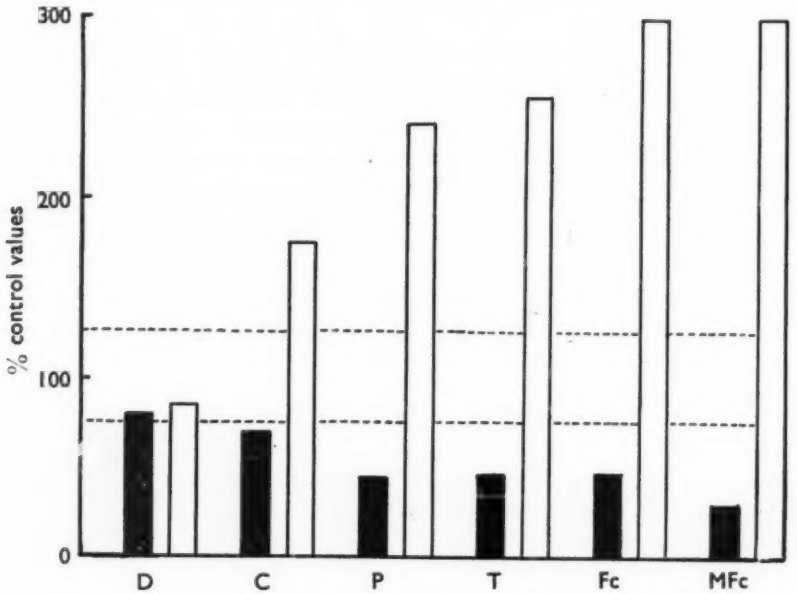

Fig. 1. Histograms to show the effects of corticosteroids (10 mg/kg for 4 days) on the histidine decarboxylase activities of rat liver (■) and pyloric stomach (□). All values are expressed as percentages of the control values. Values under 75% or over 125% denote a significant change of enzyme activity. The abbreviations are D, deoxycortone; C, cortisone; P, prednisolone; T, triamcinolone; Fc, fludrocortisone; MFc, 2-methylfludrocortisone.

enzyme activity to 287% of the control value and fludrocortisone increased it to 308%. Cortisone was less active and deoxycortone was inactive. No further changes occurred when the treatment was extended to 9 days.

The effect of exposure to cold on histamine formation

The effect of exposure to cold on the histidine decarboxylase activities of rat liver and pyloric stomach (Fig. 2) was similar to that after injections of the potent glucocorticoids. For example, after the rats had been subjected to cold for only

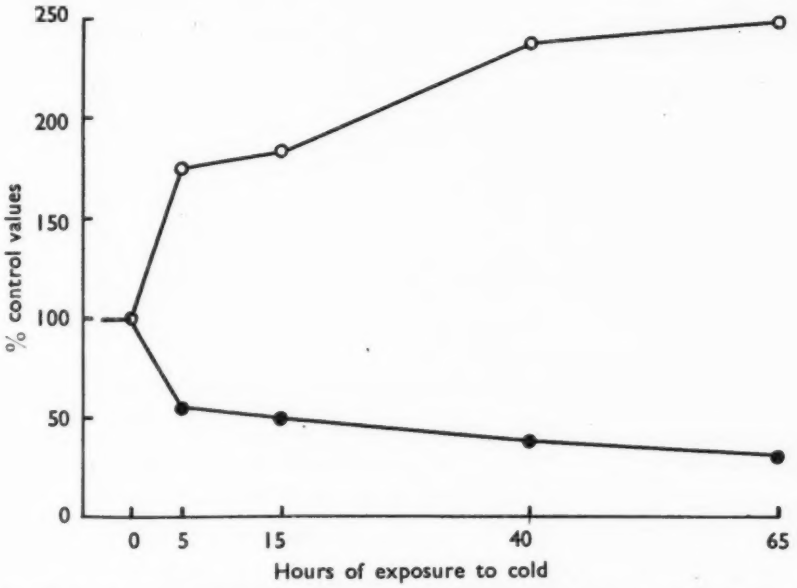

Fig. 2. The effect of exposing rats to cold for varying times on the histidine decarboxylase activities of the liver (●——●) and pyloric stomach (○——○). All values are expressed as percentages of the control values. Note that the changes in the rate of histamine formation are similar to those after glucocorticoids.

5 hr the enzyme activity of the liver was reduced to 58% of the control value, whilst that of the pyloric stomach was increased to 175%. More pronounced changes were produced on exposure to cold for longer periods.

The effect of histamine liberators on histamine formation

The effects of injections of polymyxin B and compound 48/80 on the histidine decarboxylase of the liver and pyloric stomach were similar to those found after glucocorticoids and after exposure to cold.

Short-term treatment. Whilst the rate of histamine formation by the pyloric stomach was little affected, this treatment reduced the histidine decarboxylase activity of the liver by more than 60% (Fig. 3). The enzyme activity of the liver was still reduced 3 days after the injections, and activity was not restored until after 9 days.

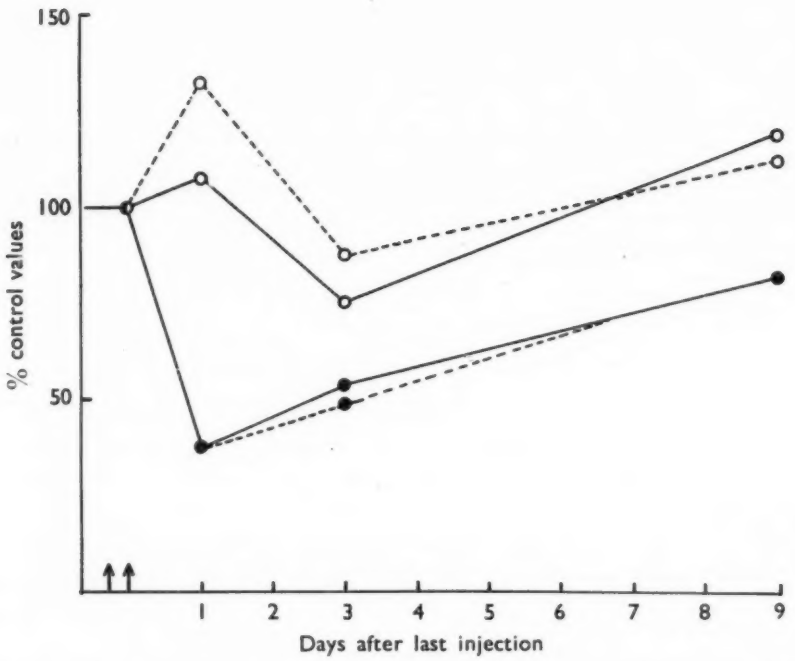

Fig. 3. The effects of treating rats with 2 doses (shown at the arrows) of polymyxin B (complete lines) and compound 48/80 (broken lines) on the histidine decarboxylase activities of the rat liver (●) and pyloric stomach (○). All values are expressed as percentages of the control values. Note that both histamine liberators reduce the rate of histamine formation in the liver, but do not significantly alter that in the pyloric stomach.

Long-term treatment. Both histamine liberators increased the enzyme activity in the pyloric stomach (Fig. 4). The activity was 157% of the control value when determined 1 day after the last injection of polymyxin B, and 149% after compound 48/80. Four days later, the activities were not significantly different from those in control rats.

In the liver, both polymyxin B and compound 48/80 reduced the histidine decarboxylase activity to about 30% of the control values. Inhibition was still marked 5 days after the last injection, and complete recovery of enzyme activity did not occur until about the 13th day.

The effect of adrenalectomy on histamine formation

The histidine decarboxylase activities of rat liver and pyloric stomach were not significantly altered after bilateral adrenalectomy or after mock-adrenalectomy.

The effects of corticosteroids and adrenalectomy on histaminase

Injections of corticosteroids did not change the histaminase activity of the rat ileum. After adrenalectomy, however, the activity was reduced. This is shown in Fig. 5 and confirms the finding of Karady, Rose & Browne (1940).

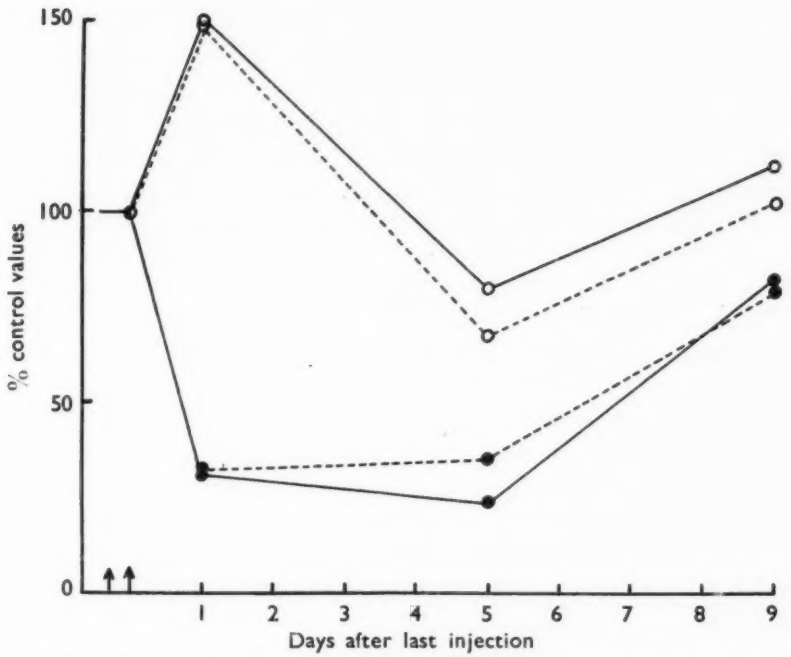

Fig. 4. The effects of treating rats with repeated doses (shown between the arrows) of polymyxin B (complete lines) and compound 48/80 (broken lines) on the histidine decarboxylase activities of the rat liver (●) and pyloric stomach (○). All values are expressed as percentages of the control values. Note that both histamine liberators reduce the rate of histamine formation in the liver but increase the rate in the pyloric stomach.

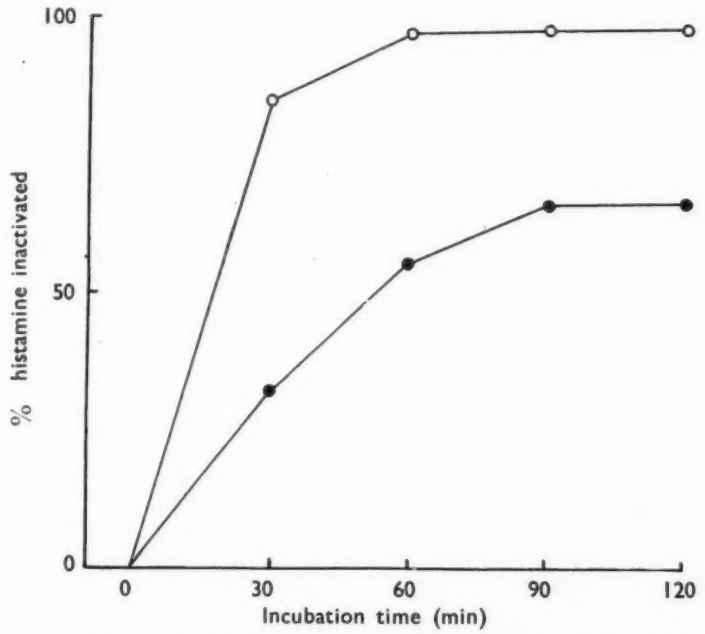

Fig. 5. The rate of destruction of histamine by the ileum of adrenalectomized rats (●——●) and mock-adrenalectomized rats (○——○). Note that adrenalectomy reduces the rate of destruction of histamine.

The effects of corticosteroids on 5-hydroxytryptamine formation

The liver and kidney are the most potent sources of 5-hydroxytryptophan decarboxylase activity in the rat (West, 1958). Corticosteroids had no effect on the activity in either of these tissues, although the 5-hydroxytryptamine contents of the skin and small intestine are considerably lowered by such treatment (Telford & West, 1960). It is of interest that corticosteroids also do not change the activity of 5-hydroxytryptophan decarboxylase in rat brain (Price & West, 1960).

DISCUSSION

We have already shown that corticosteroids reduce the histamine and 5-hydroxytryptamine contents of the rat skin and small intestine but increase the amine contents of the pyloric stomach. The ability of the steroids to produce these changes is related to their glucocorticoid activity (Telford & West, 1960). The present work shows that the changes in the histamine levels may be due to alterations in the rate of histamine formation. Thus triamcinolone, fludrocortisone, and 2-methylfludrocortisone markedly alter the histidine decarboxylase activities of the rat liver and pyloric stomach ; prednisolone and cortisone are weaker in these effects, whilst deoxycortone (a mineralocorticoid) is inactive. The present results also show that the 5-hydroxytryptophan decarboxylase activities of rat tissues are unchanged by glucocorticoid treatment, suggesting that these compounds alter the 5-hydroxytryptamine content of the tissues by means other than by inhibiting its formation.

The liver has a far greater histamine-forming capacity than any other rat tissue, and it has been suggested that it may supply the histamine requirements of most of the body (Telford & West, 1960). Inhibition of histamine formation in this tissue after treatment with glucocorticoids may thus be sufficient to account for the depletion of histamine occurring in the skin and small intestine. The present results show that the histidine decarboxylase activity of the liver is as much reduced after treatment with glucocorticoids for 4 days as after treatment for 9 days, although extensive depletion of histamine from the skin and small intestine does not occur until after treatment for 9 days. Similarly, the increase in the enzyme activity of the pyloric stomach is no greater after treatment for 9 days than after treatment for 4 days, although the increase in the histamine content of this tissue is greater after the longer period of treatment. Thus there is a time-lag before the changes in the activity of the decarboxylating enzyme are reflected by alterations in the tissue levels of histamine.

Using tracer techniques, Schayer (1956) showed that cortisone raises the histamine-binding capacity of rat pyloric stomach but lowers that of the lung and skin. Later (1957) he reported that these effects are reversed after adrenalectomy, although the reduction in the histamine-binding capacity of the pyloric stomach did not involve a decrease in histidine decarboxylase activity. In the present work, adrenalectomy failed to modify the formation of histamine by both the pyloric stomach and the liver, but there was a reduction in the histaminase activity of the ileum. Thus adrenalectomy may raise the tissue levels of histamine by means other than by stimulating its formation.

The effects of histamine liberators and of exposure to cold on the histidine decarboxylase activity of the rat liver and pyloric stomach are similar to those produced by injections of glucocorticoids. As severe stress is caused both by the treatment with histamine liberators and by exposure to cold, it is possible that the activity of the enzyme is controlled by the endogenous release of corticosteroids. Thus, severe shock occurs after injections of polymyxin B and compound 48/80, both drugs producing an abrupt fall in blood pressure and body temperature, laboured respiration, muscular weakness, and the typical symptoms of extensive histamine release. Exposure to cold produces not only hypertrophy of the adrenal gland but also an increased release of adrenocortical secretion, which in the rat is mainly corticosterone and hence glucocorticoid in nature. The present results provide further evidence, therefore, for the hypothesis of Hicks & West (1958a) that the tissue reserves of histamine are regulated by the secretion of the adrenal cortex.

More recently, Schayer, Rothschild & Bizony (1959) detected a small histidine decarboxylase activity in rat skin which was markedly increased after repeated injections of compound 48/80. Schayer (1960) has postulated that there are at least two types of histidine decarboxylase, one to produce histamine to be bound to mast cells and one to produce free histamine. In the present study, the histidine decarboxylase activity of both mast-cell-rich and mast-cell-free tissues was investigated after treatment with histamine liberators, but no relationship between enzyme activity and recovery rate of tissue histamine was found. For example, enzyme activity was not detected in skin after histamine depletion, and the enzyme activity of the duodenum (although very low) was not increased ; both skin and duodenum contain mast cells. Histamine liberators, however, markedly reduced the enzyme activity of the liver and slightly increased it in the pyloric stomach—two tissues which are nearly devoid of mast cells. It is suggested that the slow recovery of tissue histamine after extensive depletion by liberators is due in part to the prolonged inhibition of histamine formation in the liver.

The optimal conditions for the determination of histidine decarboxylase vary from tissue to tissue (Telford & West, 1961). The enzyme in the rat liver is most active at *p*H 8.0 and requires the presence of a trace of organic solvent for the detection of activity, whereas that in the pyloric stomach is most active at *p*H 7.0 and is unaffected by organic solvent. As the present results show, the enzyme in the liver differs from that in the pyloric stomach in its reaction to treatment with glucocorticoids or histamine liberators, and to exposure to cold. These findings suggest that there are two enzymes capable of decarboxylating histidine in the adult rat. The enzyme in the stomach may form histamine to act as a stimulus of gastric secretion, whilst that in the liver may be responsible for supplying histamine for storage and utilization in the rest of the animal. In the rat foetus the liver possesses considerable histidine decarboxylase activity during the latter part of pregnancy (Kahlson, Rosengren & White, 1960), and the optimal conditions for its determination in this tissue also differ from those in adult tissues (Telford & West, 1961b). Evidence is thus accumulating to indicate that there are at least three histidine decarboxylating enzymes in the rat, each producing histamine for a different function. Further studies are in progress to elucidate the nature of the intracellular decarboxylation of histidine in the rat.

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